Evidence for natural selection and barrier leakage in candidate loci underlying speciation in wood ants

Kulmuni J[1,2*], Nouhaud P[1], Pluckrose L[1#], Satokangas I[1#], Dhaygude K[1], Butlin RK[3,4]

1) Centre of Excellence in Biological Interactions, Department of Biosciences, POB 65, FI-00014, University of Helsinki, Finland
2) Tvärminne Zoological Station, University of Helsinki, J.A. Palménin tie 260, FI-10900 Hanko, Finland
3) Department of Animal and Plant Sciences, Alfred Denny Building, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK
4) Department of Marine Science, University of Gothenburg, S-405 30 Gothenburg, Sweden

#) Authors contributed equally.

Abstract

While speciation underlies novel biodiversity, it is poorly understood how natural selection shapes genomes during speciation. Selection is assumed to act against gene flow at barrier loci, promoting reproductive isolation and speciation. However, evidence for gene flow and selection is often indirect. Here we utilize haplodiploidy to identify candidate barrier loci in hybrids between two wood ant species and integrate survival analysis to directly measure if natural selection is acting at candidate barrier loci. We find multiple candidate barrier loci but surprisingly, proportion of them show leakage between samples collected ten years apart, natural selection favoring leakage in the latest sample. Barrier leakage and natural selection for introgressed alleles could be due to environment-dependent selection, emphasizing the need to consider temporal variation in natural selection in future speciation work. Integrating data on survival allows us to move beyond genome scans, demonstrating natural selection acting on hybrid genomes in real-time.

Keywords: Speciation, Hybridization, Haplodiploid, Hymenoptera, *Formica* wood ants, genomic island of divergence, genomic scan, incompatibility, gene network

Corresponding author: Jonna Kulmuni, Department of Biosciences, University of Helsinki, 00014 Helsinki, Finland, jonna.kulmuni@helsinki.fi

Short title: Candidate loci of speciation and barrier leakage

Introduction

New species are formed when populations become reproductively isolated, i.e. they do not interbreed and exchange genetic material [1]. However, until complete reproductive isolation has evolved gene flow may still occur between the diverging lineages. This genetic exchange is usually localized in the genome, with some regions of the genome resisting gene flow better than others. Regions resistant to gene flow are thought to harbor "barrier loci" that can drive the divergence between lineages despite the homogenizing effect of gene flow [2]. Barrier loci could contribute to reproductive isolation by allowing differential adaptation between diverging lineages [2]. Alternatively, barrier loci could consist of genes incompatible between the diverging lineages (Dobzhansky-Muller incompatibilities [3,4], DMIs) driven by drift or divergent selection [5].

Several recent studies have searched for barrier loci using genome scans and identified genomic islands of differentiation using various measures of genetic differentiation [e.g. refs *6–8*]. The underlying assumption in these genome scans is that the most differentiated genomic regions between hybridizing lineages are those that are resistant to gene flow and hence drive reproductive isolation. Genetic differentiation however is an indirect measure of gene flow and several problems with genome scans have been discussed [e.g. refs *9*, *10*]. The main problem lies in the fact that several other evolutionary forces (such as low recombination rate or drift) can bring about genomic regions of seemingly high differentiation between populations. Thus, we need more studies that test if natural selection is promoting speciation and halting gene flow at these genomic regions in natural populations.

Here we use ants as a model system to discover genomic regions of divergence (i.e. putative barrier loci) and to test for natural selection acting on them. In ants, males are haploid and born from unfertilized eggs from the mother, whereas females are diploid and arise via normal sexual reproduction. The haploid males thus allow the observation of natural selection acting on recessive alleles that are masked in female heterozygotes. Specifically, we use a naturally occurring hybrid population between two mound-building wood ant species, *Formica aquilonia* and *F. polyctena* from Southern Finland. These species have been estimated to diverge within the last

500, 000 years, probably at least partly in allopatry in different glacial refugia [10]. The hybrid population is well suited for unraveling the genetic basis of speciation: genetic incompatibilities segregate and persist across generations allowing to measure selection acting on these alleles in nature [11,12].

Previously, we have shown that haploid hybrid eggs (i.e. hybrid males) are laid each generation, but that males with specific introgressed alleles have low viability and die during development [12]. Yet diploid hybrid females with the same alleles persist in the population and seem to be favored over more parental-like individuals. This suggests recessive nuclear incompatibilities are in action in the haploid hybrid males, but these are masked in the diploid hybrid females where heterozygotes for introgressed alleles are favored [12]. Modeling work suggests that these opposite selective pressures between males and females can lead to purging of incompatibilities or their long-term maintenance depending on their initial frequencies, strength of selection and recombination [13]. The age of the hybrid population is unknown, but present-day individuals are not first-generation hybrids. Instead, the study population divides into two genetic lineages in a very similar way to the swordtail fish [14]; within the hybrid population one lineage is genetically more similar to one of the parental species, *Formica aquilonia* (previously referred to as W group), and the other is closer to the other parental species, *F. polyctena* (previously referred to as R group) [15]. Even though both lineages are of hybrid origin they show signs of reproductive isolation, as 94.5 % of successful matings are within a lineage [11,12] and we have never observed reproductive adult F1 individuals in nature. The *Formica* system differs from the genetic caste determination system observed e.g. in *Pogonomyrmex* harvester ants [16], because in *Formica* both queens and workers arise from the same mating events and show no genetic difference in contrast to *Pogonomyrmex*. Both lineages have previously lacked adult males with specific introgressed alleles (i.e. heterospecific alleles) or had them at low frequency due to negative selection, while the same alleles were favored in females as heterozygotes. This has resulted in strong allele frequency differences between sexes at specific marker loci, females having frequencies as high as 0.4 while males lack these alleles altogether [11]. We have shown that this antagonistic selection acting on introgressed alleles is stronger in the *F. polyctena*–like lineage, where over 90 % of male eggs

133 were estimated to die [12]. Selection is weaker on the males of the *F. aquilonia*–like
134 lineage, as is the evidence for selection favoring female heterozygotes.
135
136 Here we discover and annotate candidate barrier loci, i.e. the genomic regions that
137 associate with hybrid male breakdown and thus act as barriers for gene flow between
138 the two wood ant species. We then test if natural selection is acting on these candidate
139 barrier regions in nature using SNP genotyping and survival analysis. This allows for
140 testing the role of natural selection in genomic islands of divergence and reproductive
141 isolation within a natural population, which has not been possible in most of the
142 genomic studies of speciation.
143
144 **Results**
145
146 *Multiple barrier loci throughout the genome are candidates for hybrid male*
147 *breakdown*
148 We used pooled genomic sequencing to discover the genomic regions associated with
149 hybrid male breakdown (see Figure 1 for experimental design). We sequenced four
150 samples, each consisting of 24 individuals: *F. polyctena*–like males, *F. polyctena*–like
151 females (unmated queens), *F. aquilonia*–like males and *F. aquilonia* –like females
152 (unmated queens). These individuals were classified into lineages based on six to
153 seven diagnostic microsatellite alleles [11]. Due to haplodiploidy, this resulted in 24
154 and 48 chromosomes sampled for male and female pools, respectively. These pools
155 represent population samples and came from two to four nests each, all of which
156 belong to the same supercolony, where nests have hundreds of reproductive queens
157 and relatedness approaching zero. Our previous [11,12] and current analyses (see
158 Results) show there is no differentiation between nests within a lineage, justifying
159 pooling of individuals from different nests. We sequenced each pool with 100 bp
160 paired-end reads on its own lane in Illumina HiSeq2000 and made a *de novo* assembly
161 from each pool. Subsequently, the assembly of *F. polyctena*–like males was used as a
162 reference (genome size = 222.6 Mb, N50 = 1748 bp, 327,480 total scaffolds). Each
163 pool was mapped to the reference assembly and SNPs were called after filtering. We
164 retained 166,167 bi-allelic SNPs displaying coverage between 20 and 60 in each pool
165 and minor allele count of four over all pools.
166

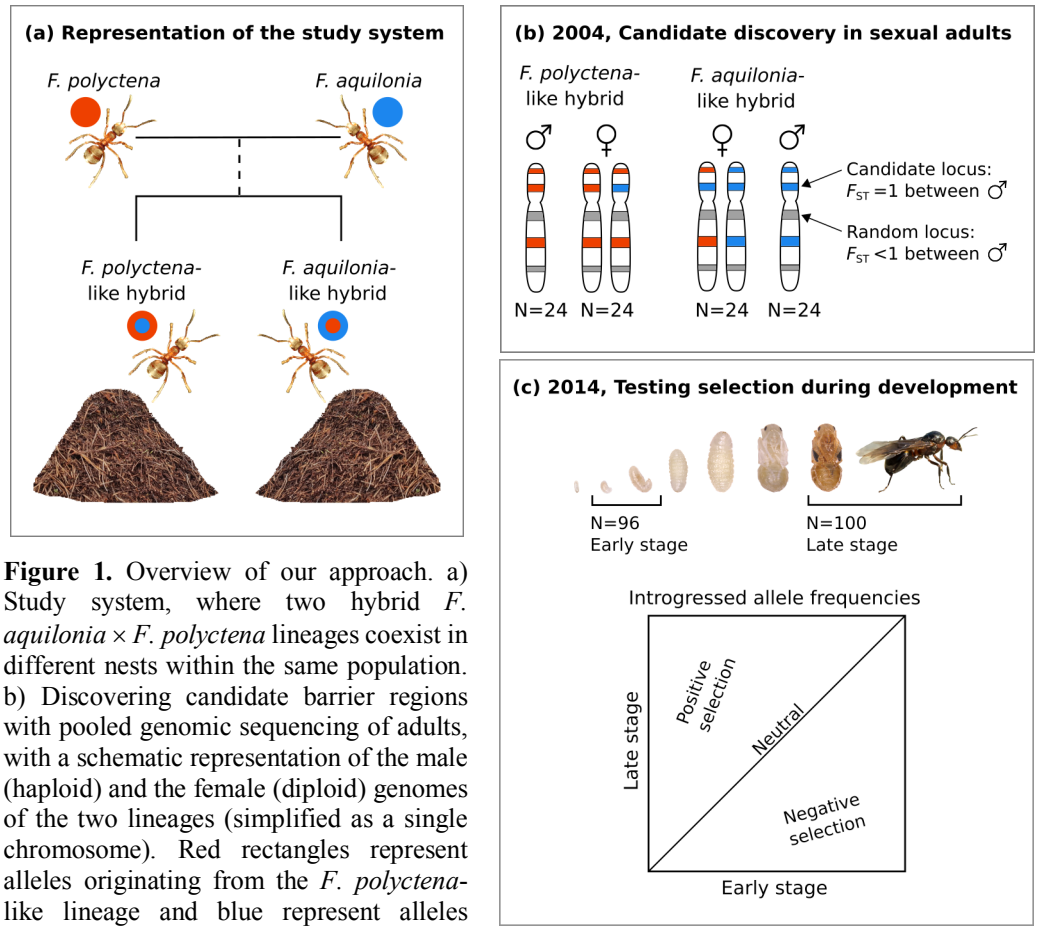


Figure 1. Overview of our approach. a) Study system, where two hybrid *F. aquilonia* × *F. polyctena* lineages coexist in different nests within the same population. b) Discovering candidate barrier regions with pooled genomic sequencing of adults, with a schematic representation of the male (haploid) and the female (diploid) genomes of the two lineages (simplified as a single chromosome). Red rectangles represent alleles originating from the *F. polyctena*-like lineage and blue represent alleles originating from *F. aquilonia*-like lineage. c) Testing natural selection at candidate barrier loci between larval (Early) and adult stages (Late).

We defined barrier loci as SNP positions where the male genomes from the *F. aquilonia*–like and *F. polyctena*–like lineages were fixed differently (i.e. F_{ST} = 1, see Supplementary Figure 1). This criterion is arbitrary in the sense that many loci showing significant differentiation (but not fixed) could be under selection too. However, loci fixed differently in males, despite shared hybrid history and variable in females are expected to experience the strongest antagonistic selection in the current population and offer the best opportunity to test for selection acting on barrier loci. We assumed that the allele fixed in *F. aquilonia*–like males represents the parental *F. aquilonia* allele and the allele fixed in *F. polyctena*–like males represents parental *F. polyctena,* from lineages that gave rise to the hybrid population.

The mean F_{ST} estimate between the *F. aquilonia*–like and *F. polyctena*–like lineages was 0.14 (s.e. = 4.66×10^{-4}) between the males, and 0.07 (s.e. = 2.27×10^{-4}) between the female pools. We found 711 SNPs (0.43 % of the total) alternatively fixed between

208 males of the *F. aquilonia*–like and *F. polyctena*–like lineages, but no fixed differences
209 between the female pools. As expected under antagonistic selection, SNPs fixed
210 differently in males were polymorphic and had mean F_{ST} of 0.34 (s.e. = 5.83×10^{-3},
211 Supplementary Figure 1) in the females. The alternatively fixed SNPs between the
212 male pools represent genomic regions of high differentiation between the two male
213 lineages despite their shared hybrid history, and thus mark candidate barrier loci that
214 are predicted to contribute to reproductive isolation and speciation. These 711 SNPs
215 fell into 610 assembled contigs (cumulative size: 2.09 Mb, 0.94% of the genome),
216 whose lengths vary between 515 – 23,815 bp (mean = 3430 bp). Seventy-eight
217 contigs had more than two barrier SNPs (maximal number of barrier SNPs on a single
218 contig was 5).
219
220 We were interested in how these candidate barrier loci locate in the genome, because
221 this can provide information about the origin of reproductive isolation and play a role
222 in how efficient the barrier loci are in maintaining divergence despite hybridization
223 and gene flow. If candidate barrier loci are co-localized in the genome their origin and
224 maintenance could be explained by selection on a few genomic regions that could, for
225 example, harbor structural re-arrangements. Alternatively, candidate barrier loci
226 scattered across the genome could effectively reduce gene flow over much of the
227 genome. We used the genome of the closely-related *Formica exsecta* [17] (277 Mb,
228 14,617 scaffolds, N50 = 997,654), which is better assembled compared to the poolseq
229 genome assembly, to map the location of candidate barrier loci. *F. exsecta* is
230 estimated to have diverged from the parental species of our hybrid population 5 Mya
231 [18]. We used the 610 contigs containing candidate barrier SNPs as queries and
232 searched the *F. exsecta* genome assembly using BlastN. If our query scaffold was
233 larger than 5, 000 bp we used +/- 2, 500 bp surrounding the candidate barrier SNP as
234 query. Our candidate barrier loci map to a total of 134 *F. exsecta* contigs, with a
235 cumulative coverage of 56.2 % of the *F. exsecta* genome, both of which are
236 significantly less than for a similar number of random SNPs in our data set
237 (Supplementary Figure 2). Haploid chromosome number varies between 26 and 28 in
238 *Formica* [19]. Assuming evenly-sized chromosomes our candidate barrier loci are
239 likely to be situated in over 10 chromosomes.
240

241 *Candidate barrier loci are predicted to interact at the protein level*
242 Next, we were interested in the possible functions of our candidate barrier loci, as
243 they can be informative about the putative selection pressures and mechanisms of
244 hybrid male breakdown. We annotated each of the 610 contigs containing candidate
245 barrier SNPs, first extracting the transcripts of the genes located in those regions from
246 the closely related *F. exsecta*. Out of 571 candidate barrier regions (see Material and
247 Methods; each region was defined as 5, 000 bp around the candidate barrier SNP) that
248 we were able to anchor in *F. exsecta* (all blastn *e*-values < 10^{-141}), we recovered 590
249 unique genes. Sixty-three of the candidate genomic regions did not contain any
250 annotated gene. The mean number of genes per candidate genomic region was 1.4,
251 and the maximum was six. For each gene, its *F. exsecta* transcript sequence was
252 recovered and blasted (blastx) against *Drosophila melanogaster* r6.15 proteins
253 recovering 317 genes, whose homologs reside in candidate barrier regions
254 (Supplementary Table 1).
255
256 The genetic model for hybrid breakdown suggests inviability and sterility are caused
257 by dysfunction and negative epistatic interactions between diverged genes from the
258 parental species. One possible mechanism that can result in epistasis are molecular
259 interactions. We thus asked if there was any evidence for molecular interactions in the
260 form of protein-protein interactions between the gene products in candidate barrier
261 regions. For this we used the recovered *D. melanogaster* proteins and the STRING
262 database [20], which contains information on known and predicted protein-protein
263 interactions. We found that candidate barrier regions harbor proteins that show
264 significantly more interactions with each other than a random set of proteins drawn
265 from the *D. melanogaster* genome (PPI enrichment *p*-value = 9.65×10^{-13}, Figure 2).
266 Out of 268 genes annotated and having information in the STRING database, 167
267 genes fall into a single interaction network, with evidence for protein-protein
268 interactions in *D. melanogaster* or other species. Seventy-four of the network genes
269 had evidence for interactions which were experimentally determined or obtained from
270 curated database (PPI enrichment *p*-value = 0.03). However, randomly drawing 711
271 SNPs from our pooled sequencing data 1000 times showed that similar enrichment in
272 protein-protein interactions (i.e. network) could be retrieved for a random set of SNPs
273 as well. This suggests either a bias towards functional and interacting sets of genes in

our total SNP dataset or a bias in our method towards interacting sets of genes arising due to conservation (only genes conserved between ants and *D. melanogaster* can be included in the analysis). However, this does not mean the interactions which were documented would be unreliable. In summary, our results suggest interactions among the genes in candidate barrier regions, but these interactions are not significantly more abundant than for random sets of SNPs in our data.

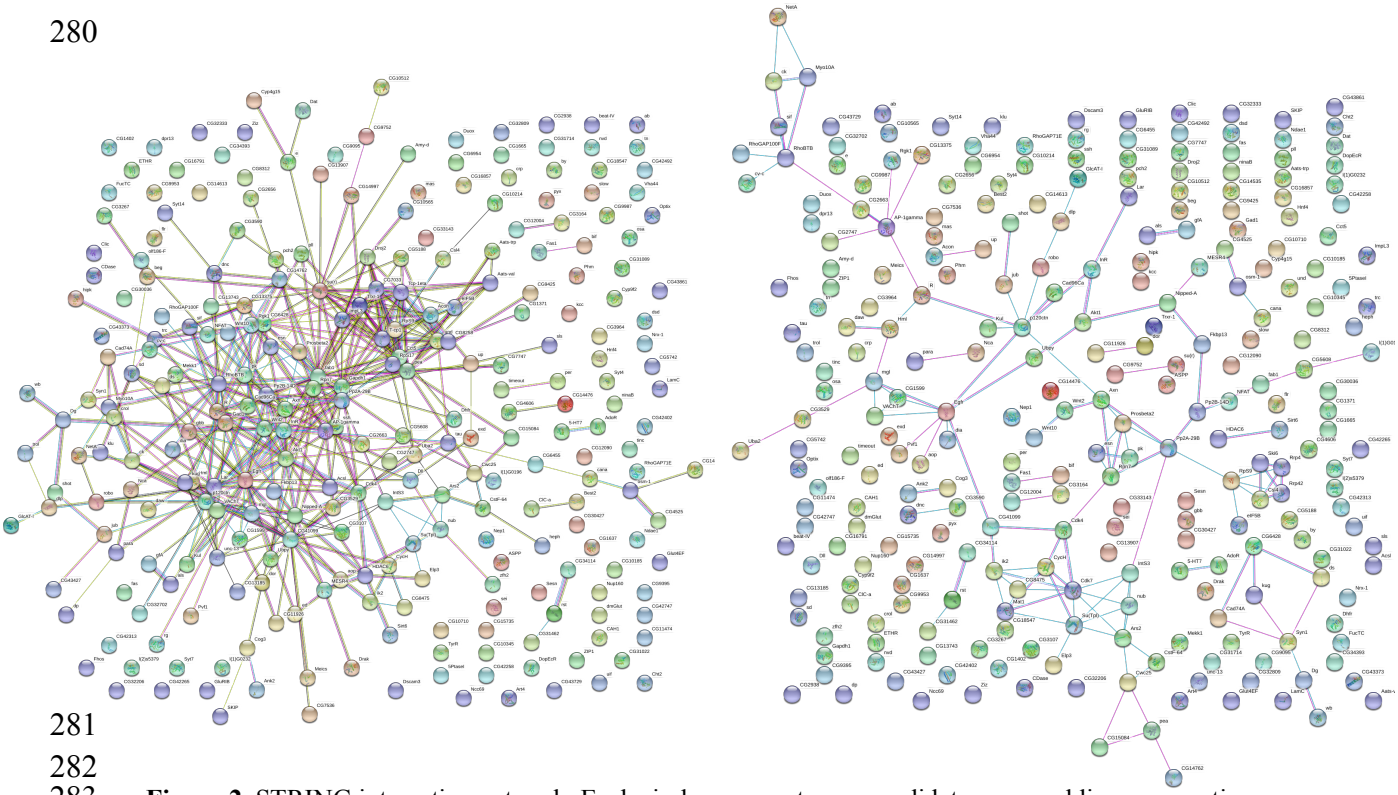
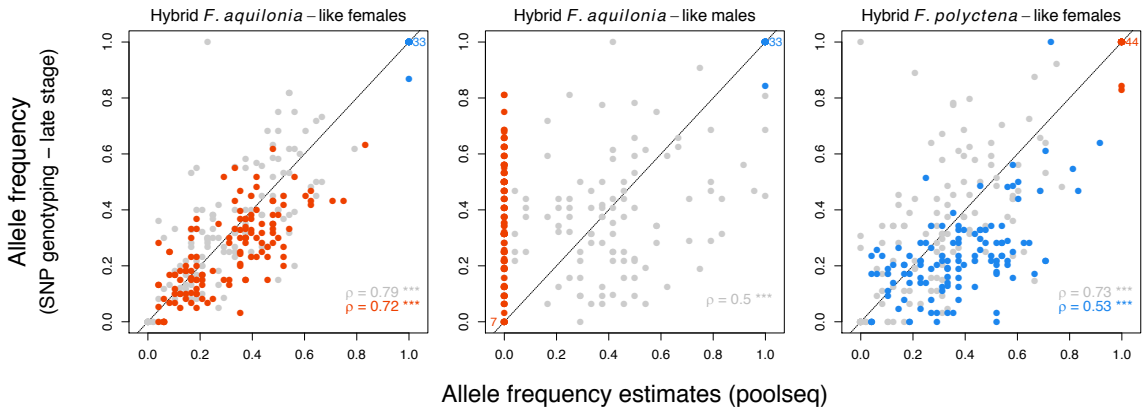


Figure 2. STRING interaction network. Each circle represents one candidate gene and lines connecting them indicate support for interaction between the genes. Majority of interactions fall into a single network. Line color indicates following: blue = data from curated databases, pink = experimentally determined interactions, black = co-expression. Analysis is based on *D. melanogaster* homologs. Left panel: all interactions, and right panel: experimentally determined interactions or interactions obtained from curated databases.

Next, we were interested in the functions of genes within candidate barrier regions. For this we used the *D. melanogaster* gene IDs. The top three biological processes characterizing our candidate barrier genes are "developmental process" ($p = 3.5\times10^{-18}$), "system development" ($p = 3.5\times10^{-18}$), and "single-organism developmental process" ($p = 4.03\times10^{-18}$). Similarly, the top three molecular functions are "ion binding" ($p = 8.55\times10^{-6}$), "protein binding" ($p = 3.67\times10^{-5}$) and "calcium ion binding" ($p = 8.74\times10^{-5}$). These biological processes and molecular functions were highly significantly enriched when compared to the genome average of *Drosophila melanogaster*. However, they were not significantly enriched if compared to a random

299 set of SNPs drawn from our data, as all of them are found in over 5 % of our
300 simulations.
301
302 *Testing for signs of selection at candidate barrier loci in a natural population*
303 We tested for selection during development by genotyping altogether 96 individuals
304 at early (larva) and 100 individuals at late (adult or late stage pupa) developmental
305 stages (Supplementary Table 2), with 180 random and 183 candidate barrier SNPs
306 (Figure 1). Out of these, 163 candidate barrier SNPs and 137 random SNPs were
307 successfully genotyped. The 137 random SNPs were located on 136 contigs
308 (cumulative size: 484 kb, 0.22% of the genome), whereas the 163 candidate barrier
309 SNPs located on 159 contigs within our assembly (cumulative size: 523 kb, 0.23% of
310 the genome). Individuals used in genotyping were collected in Spring 2014 from the
311 same hybrid population as the one sampled for the pooled sequencing analysis ten
312 years earlier.
313
314 The SNP genotyping confirmed that within the hybrid population, *F. polyctena*–like
315 and *F. aquilonia*–like lineages are genetically distinct from one another
316 (Supplementary Figure 3). We found two small female larvae, which were genetically
317 intermediate and had unique combinations of putative parental alleles from the two
318 lineages suggesting they were early-generation hybrids between the lineages
319 (Supplementary Figure 3). The *F. polyctena*–like individuals, and especially the
320 males, were previously shown to experience strongest selection. Unfortunately, we
321 were able to sample only 8 males from this lineage, five of which turned out to be
322 diploid, thus *F. polyctena*–like males were excluded from the analysis. The following
323 analyses were performed for *F. polyctena*-like females, *F. aquilonia*-like males and *F.*
324 *aquilonia*-like females.
325
326 First, we asked if adult allele frequencies inferred from the pooled sequencing
327 correlated with adult allele frequencies estimated from the SNP genotyping data, as
328 the two data sets have been collected ten years apart and allele frequencies were
329 estimated in different ways. Over all the genotyped SNPs, allele frequencies between
330 the two data sets correlated well in *F. polyctena*–like females (Spearman's
331 correlation, $\rho = 0.87$, $CI_{95} = [0.82, 0.90]$, $p < 0.001$) and in *F. aquilonia*–like females

332 (ρ = 0.91, CI_{95} = [0.88, 0.94], $p < 0.001$) (Figure 3). The correlation was lower for the
333 males with a Spearman's correlation coefficient of 0.71 (CI_{95} = [0.64, 0.76], $p <$
334 0.001) between adult allele frequencies estimated from pooled sequencing and
335 genotyping (Figure 3). The lower correlation in males compared to females can be
336 explained, at least in part, by the lower number of sampled chromosomes for
337 (haploid) males compared to (diploid) females creating a larger error in the estimated
338 allele frequencies. Overall, correlations between allele frequency estimates are in line
339 with other studies focusing on the accuracy of allele frequency estimates derived from
340 poolseq [21] and suggest pooled sequencing and SNP genotyping gave similar results.
341



342
343 **Figure 3.** Allele frequencies correlate between pooled sequencing (adults sampled in 2004) and SNP
344 genotyping in late stage (adults sampled in 2014). Putative barrier loci are colored according to their
345 polarization (red: allele introgressed from *F. polyctena*-like to *F. aquilonia*-like, blue: allele introgressed
346 from *F. aquilonia*-like to *F. polyctena*-like). Control SNPs (in grey) are polarized by the minor allele in
347 the poolseq data. Linear regressions are indicated for each class of SNPs according to their colors, along
348 with their Spearman's correlation coefficients and their significance (***: $p < 0.001$).
349

350 *Candidate barrier SNPs show significant heterozygote excess in females*

351 Next, we tested if the barrier loci showed significant allele or genotype frequency
352 changes during development from larva to adult compared to the random loci. This
353 would demonstrate natural selection acting on the candidate barrier loci in nature. No
354 significant allele frequency change was observed between larval and adult stage at
355 candidate barrier loci compared to random loci in females of *F. aquilonia*– or *F.*
356 *polyctena*–like lineage (Figure 4, Figure 5).
357

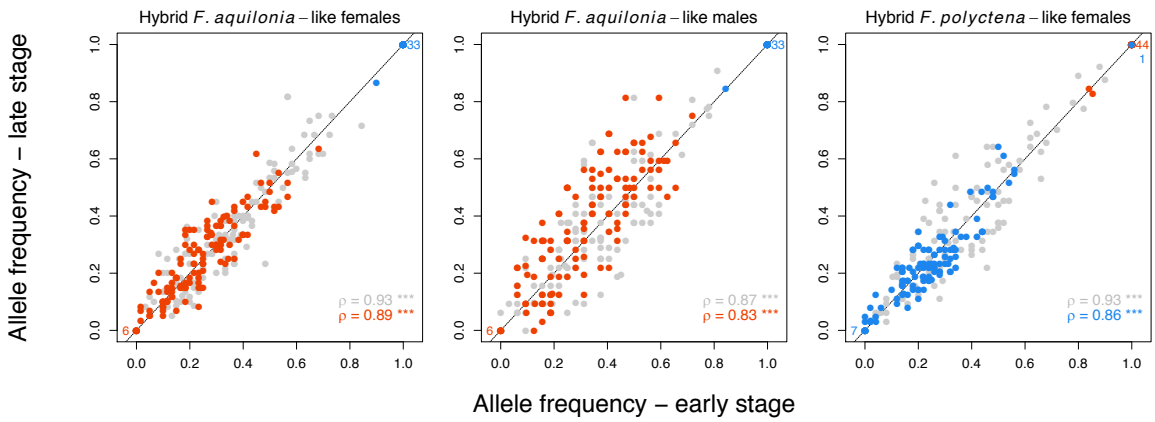


Figure 4. Allele frequency correlation between early (larva) and late (adult) developmental stages in random (grey) and candidate barrier loci (red: allele introgressed from *F. polyctena*-like to *F. aquilonia* -like, blue: allele introgressed from *F. aquilonia*-like to *F. polyctena*-like) in year 2014. No strong allele frequency change was observed from larva to adult in females, but frequencies in males were significantly different between larva and adult (see results). Numbers in bottom left and upper right parts indicate the number of candidate barrier SNPs fixed. Linear regressions are indicated for each class of SNPs according to their colors, along with their Spearman's correlation coefficients and their significance (***: $p < 0.001$).

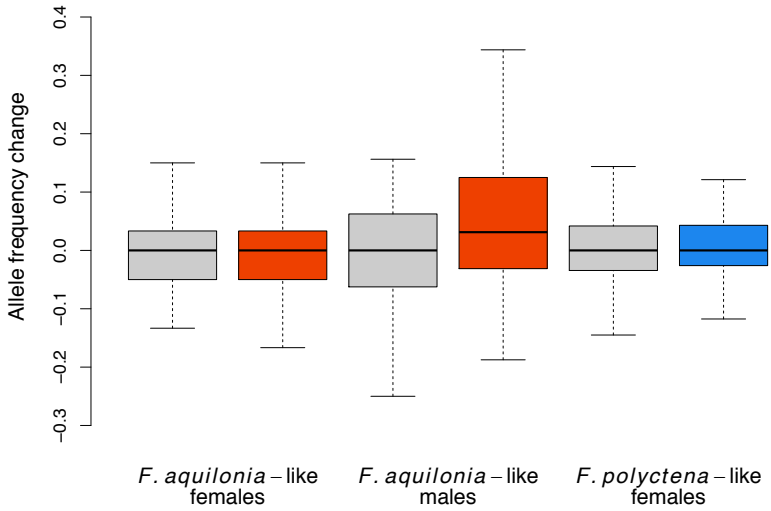


Figure 5. Mean allele frequency change between early (larva) and late (adult) developmental stages in random (grey) and candidate barrier loci (red or blue) in year 2014. Allele frequencies at barrier loci are polarized according to the introgressed allele. Candidate barrier SNPs have significantly stronger allele frequency change compared to random SNPs in *F. aquilonia*–like males (p = 0.003, see methods for details on the model).

Our previous microsatellite-based study showed that introgressed alleles were favored in females when heterozygous. This selection acted throughout the female lifetime. Thus, we searched for signs of similar selection testing for increase in heterozygote excess between early and late stages using F_{IS} estimates. Indeed, there was a tendency for more negative mean F_{IS} values (i.e. greater excess of heterozygosity) in candidate

barrier loci at the adult stage (*F. aquilonia*–like F_{IS} = –0.116, *F. polyctena*– F_{IS} = –0.2) compared to the larval stage (*F. aquilonia*–like F_{IS} = –0.085, *F. polyctena*– F_{IS} = –0.179) in both female lineages (Figure 6), but this tendency was not statistically significant in either lineage when compared to random loci (*F. aquilonia*–like p = 0.295, *F. polyctena*–like p = 0.643). However, candidate barrier loci had significantly greater heterozygote excess compared to random loci already at larval stage (GLMM, z-value = –4.404, $p < 0.001$) and this pattern remained at adult stage as well (GLMM, z-value= –7.133, $p < 0.001$) in *F. polyctena*–like females. To account for possible effects of linkage disequilibrium, the SNP data set was pruned by omitting SNPs closer than 50 kb, 100 kb or 500 kb according to their BLAST hits in the *F. exsecta* assembly. Pruning did not affect the above results (all p -values reported above from GLMMs remained significant for all pruning intervals; Supplementary Figure 8, panels A-D). Greater excess heterozygosity in candidate barrier SNPs compared to random SNPs can result from either (i) strong allele frequency differences between parental genotypes or (ii) selection for heterozygosity that has already acted at candidate barrier loci before the larval stage we sampled (i.e. between egg and larval stage).

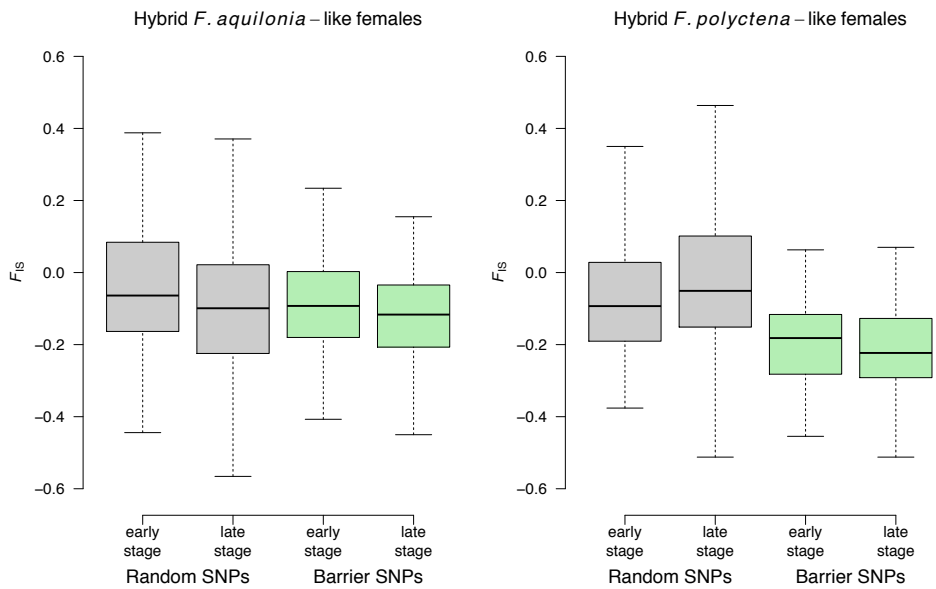


Figure 6. Mean F_{IS} estimates in early (larva) and late (adult) developmental stages in random (grey) and candidate barrier loci (green) in year 2014.

404 *Evidence for selection in candidate barrier SNPs in F. aquilonia–like males*
405 Then, we tested for allele frequency changes in *F. aquilonia*–like males, expecting the
406 introgressed alleles to decrease in frequency during development as shown in our
407 previous microsatellite based study [12]. Allele frequencies were significantly
408 different between larval and adult developmental stages in candidate barrier SNPs
409 compared to the random SNPs (glmer, *z*-value= –2.98, p = 0.00286), the candidate
410 barrier SNPs showing more frequency change during development (Figure 5).
411 However, the frequencies of the introgressed alleles increased from larval to adult
412 developmental stages in *F. aquilonia*–like males (Figure 4), a pattern opposite to our
413 expectations. Significant increase of introgressed alleles at candidate barrier loci in
414 contrast to alleles at random loci suggests selection favored the introgressed alleles in
415 *F. aquilonia*–like males during development. Again, this result remained significant
416 after pruning the SNP data (Supplementary Figure 8, panel E). Unfortunately, we
417 were unable to test selection acting at candidate barrier loci in *F. polyctena*–like
418 males, the lineage where we had the strongest expectation of negative selection,
419 because too few *F. polyctena*–like males were sampled from the population. The low
420 occurrence of these males is consistent with their estimated high mortality [12].
421
422 *Barrier leakage and significant variation in the strength of barriers through time*
423 Introgressed (i.e., *F. polyctena*–like) alleles were absent from adult *F. aquilonia*–like
424 males at 163 candidate barrier loci in year 2004 (allele frequency of zero in the
425 poolseq data). However, in year 2014 significant proportion of these loci harbored
426 alleles introgressed from the *F. polyctena*–like lineage (123 loci, i.e. 75.5%) to *F.*
427 *aquilonia*–like males. The mean frequency of introgressed alleles in these males was
428 0.36, the most common introgressed allele being at a frequency of 0.81. Sampling
429 different sub-populations within a lineage at different years is unlikely to drive
430 increase of introgressed allele frequencies between 2004 and 2014 in our samples of
431 *F. aquilonia*–like males: We observed no population substructure within the *F.*
432 *aquilonia*–like lineage in 2005 or 2014 (Supplementary Figures 3 and 4). Increase of
433 introgressed alleles in males is not likely to be caused by errors in male poolseq-based
434 allele frequency estimates either. All but one of the candidate barrier SNPs (33 out of
435 34 SNPs) that were initially fixed in both adult males and females of the *F.*
436 *aquilonia*–like lineage in the pooled sequencing data (year 2004) were still fixed in
437 the genotyping data (year 2014), showing consistency across data sets. If differences

438 between allele frequency estimates from 2004 and 2014 were caused by errors in
439 poolseq allele frequency estimates, this should affect all SNP loci equally. However,
440 this is not the case, as only candidate barrier SNPs that were polymorphic in females
441 of the *F. aquilonia*–like lineage have now "leaked" into *F. aquilonia*–like males. Our
442 results thus suggest that in year 2014 a proportion of the barrier loci are "leaking" in
443 *F. aquilonia*–like males. At these loci introgressed alleles were on average selected
444 for in males as evidenced by significant frequency increase between larval and adult
445 stages (see above), a pattern opposite to our previous observations [11,12].
446
447 Using microsatellite genotyping, we further verified that increasing frequencies of
448 introgressed alleles at candidate barrier SNPs in *F. aquilonia*–like males did not
449 represent technical artifacts. Specifically, we genotyped the individuals collected in
450 2014 and used in SNP genotyping with nine previously used microsatellite markers
451 [5,12] and compared them to samples collected in 2004, 2008 and 2011
452 (Supplementary Table 3). Microsatellite allele frequencies from 2014 paralleled the
453 pattern observed in candidate barrier SNPs in the same year. Indeed, *F. aquilonia*–like
454 males in 2014 harbor microsatellite alleles introgressed from the *F. polyctena*–like
455 lineage that were missing from them in 2004 (Table 2). Specifically, the frequencies
456 of two microsatellite alleles ($Fy3_{190}$ and $Fy15_{222}$) introgressed from *F. polyctena* to *F.*
457 *aquilonia*–like males have fluctuated over the years from 0 in 2004 to 0.28 in 2014
458 for $Fy15_{222}$ and from 0 in 2004 to 0.45 in 2014 for $Fy3_{190}$ (Supplementary table 3,
459 Figure 7A). No leakage of alleles introgressed from the *F. aquilonia*-like lineage to
460 the *F. polyctena*–like males was observed, the five introgressed microsatellite alleles
461 remaining at a null frequency in adult *F. polyctena*–like males in 2004 (N=35), 2008
462 (N=41) and 2011 (N=23, Supplementary table 3).
463
464
465 **Discussion**
466
467 Several recent genome scan studies have used next-generation sequencing to map
468 genomic regions putatively under divergent natural selection and underlying
469 reproductive isolation (reviewed in [2]). Approaches can often be based solely on
470 comparing genomes, because observing natural selection, collecting data on fitness or
471 molecular characterization of loci underlying reproductive isolation is challenging.

472 However, several suggestions have been made to move beyond genome scans with an
473 integrative approach [2,22]. To bridge the gap between genotype and fitness in
474 genome scan studies we first took advantage of male haploidy in ants and discovered
475 genomic regions of high divergence (i.e. candidate barrier regions). We then tested if
476 natural selection is acting on these genomic regions in a natural population using a
477 SNP panel and survival analysis, expecting haploid males to show the strongest
478 signals of selection against gene flow at barrier loci. However, instead we document
479 signature of barrier leakage at the candidate loci, with significant increase in the
480 frequency of introgressed alleles in *F. aquilonia*–like males during development.
481 Below we discuss reasons for the signatures of barrier leakage including environment-
482 dependent incompatibilities, formation of compatible combinations of parental alleles
483 and recombination breaking association between selected and hitchhiking loci. The
484 ant system is dynamic and allows further investigations into how selection acts on
485 hybrid genomes in cases of speciation with gene flow.
486
487 *Considering barrier loci that evolve in a network*
488 Hybrid breakdown is expected to result from deleterious epistatic interactions (i.e.
489 intrinsic incompatibilities) between diverged genes of the parental species [3,4].
490 Relatively few studies have investigated the effect of incompatibilities in networks or
491 incompatibility loci with more than one interaction (in comparison to two-locus
492 DMIs) for speciation, but see [5,23–25]. This is in stark contrast with our
493 understanding of molecular biology: genes and their products interact with several
494 other genes, are part of gene interaction networks, and are regulated by transcription
495 factors. We found that candidate barrier and hitchhiking loci likely cover a large
496 proportion of the genome in two *Formica* ant species, which diverged within the last
497 500 000 years [10]. We also found support for multiple protein-protein interactions
498 between genes located among the candidate barrier regions. Majority of genes in these
499 regions were predicted to form a single protein-protein interaction network, with
500 interactions for homologous proteins documented or predicted in other species.
501 However, our bootstrap analysis shows that the network found among the candidate
502 barrier loci was not significantly different from the genomic background: any
503 polymorphisms we were able to call within our pooled sequencing data was likely to
504 give significant enrichment of protein-protein interactions using our analysis pipeline.
505 Our annotation is based on *D. melanogaster* homologs, which makes our pipeline

506 biased towards conserved genes. If conserved genes are also more likely to have
507 evidence for protein-protein interactions (as suggested by some studies [26]), that
508 could lead to enrichment of interactions among both candidate barrier loci and
509 random sets of SNPs with our data and pipeline. Our results highlight potential biases
510 when extrapolating data from model organisms into non-model systems.
511 Bootstrapping analyses, such as presented here, can reveal that candidate genes are
512 not any different from genomic background of the focal species. However, this does
513 not mean that the interactions we found would be unimportant. Protein-protein
514 interactions could still create epistatic interactions among the barrier loci. Our results
515 are consistent with the idea of multiple genes underlying hybrid breakdown that
516 interact in a network instead of multiple two-locus interactions. Functional and
517 population studies in *Formica* are needed to test if mismatching combinations of
518 parental alleles at network loci lead to hybrid inviability, as predicted if protein-
519 protein interactions cause negative epistasis [3,4].
520
521 Annotation of candidate barrier regions suggests genes in these regions play a role in
522 development, with 133 genes out of 268 investigated having the GO term
523 "developmental process". These results are consistent with our previous findings
524 where we show hybrid male breakdown during development in the *F. polyctena*–like
525 lineage [12]. Furthermore, developmental problems are a common cause of hybrid
526 breakdown [27,28]. Our analysis pipeline is likely to produce a bias towards
527 conserved genes because it is based on *D. melanogaster* homologs, and
528 developmental genes are more likely to be conserved at the sequence level [29]. Thus,
529 it is likely that additional gene functions could be found if we were able to annotate
530 the function of all open reading frames within candidate barrier regions. Interestingly,
531 our candidate barrier loci included homologs of two major barrier loci in *Heliconius*:
532 a member of the *Wnt* gene family and *optix,* which are known to act as barriers to
533 gene flow and are responsible for variation in wing patterning between *Heliconius*
534 races [6].
535
536 But why would a developmental gene network have diverged between the two
537 *Formica* species parental to the hybrids? Divergence could have been driven by drift
538 or differential adaptation between the parental species. The parental species have
539 likely diverged in allopatry and may now be adapted into different climatic

540 conditions, *F. aquilonia* being distributed in Northern Eurasia and higher altitudes of
541 Central Europe, while *F. polyctena* is likely adapted to warmer climate and occurs in
542 Central Europe and Southern parts of Fennoscandia and Russia [30]. While the
543 species have adapted into different climatic regimes it is plausible that their
544 developmental programs have adapted and diverged too. However, developmental
545 programs can diverge also at least partly by drift. Developmental system drift is a
546 common phenomenon [31] where homologous characters develop via different
547 regulation of genes or involve completely different sets of genes.
548
549 *Signs of selection at candidate barrier loci and barrier leakage*
550 Previously we have shown antagonistic selection on ant hybrids, where introgressed
551 alleles were favored in female heterozygotes, but selected against in haploid males,
552 with strongest evidence of this in the *F. polyctena*–like lineage [11,12]. Our current
553 dataset shows a trend of increasing heterozygosity excess at candidate barrier loci
554 during female development, but this is not statistically significant after removal of
555 two outlier individuals likely to be early generation hybrids. However, we find a
556 significant excess of heterozygosity at the candidate barrier loci compared to the
557 random loci. This excess heterozygosity is likely to be caused by selection either in
558 the current generation or earlier in population history. First, strong allele frequency
559 differences between mothers and fathers would result in high heterozygosity in
560 diploid females without the action of selection in the sampled generation. However, in
561 this scenario initial differentiation between sexes at candidate barrier loci was likely
562 caused by selection acting in earlier generations (see below). Second, selection that
563 favors heterozygotes may have already acted on the candidate loci before the larval
564 stage we sampled. Our previous study documented selection during development
565 using eggs and few days old larvae and compared them to adults, whereas here we use
566 1-2 week old larvae and adults. Thus, in the current study we are likely to miss any
567 signal of selection acting earlier in development.
568
569 We assumed the candidate barrier loci to represent recessive incompatibilities and loci
570 linked to them, thus expecting to find evidence of negative selection in the haploid
571 males [11,12]. However, we find the opposite with introgressed alleles increasing in
572 frequency at the candidate barrier loci in the *F. aquilonia*–like males during
573 development. This is consistent with natural selection favoring introgression in these

574 males. The magnitude of change is comparable to that observed within a generation in
575 other systems, e.g. in stick insects for the color pattern locus [32].
576
577 Selection for introgression within a generation in males may be connected to patterns
578 we observed between years: A proportion of the barrier loci showed barrier leakage
579 resulting in increase of introgressed alleles in males sampled 10 years apart. These
580 rising frequencies of introgressed alleles in *F. aquilonia*–like haploid males between
581 years can be explained by at least three hypotheses, which are not mutually exclusive.
582 First, the barriers to gene flow can be gradually breaking down in *F. aquilonia*–like
583 males, which could explain both within- and between-year patterns. Barrier breakage
584 could be due to recombination and subsequent formation of compatible combinations
585 of barrier loci in males. Barriers for gene flow could also break down due to relaxed
586 selection. Under this model the haplodiploid systems (and X-chromosomes) can show
587 fluctuating allele frequencies at candidate barrier loci, comparable to those observed
588 in our data, if sexes had different allele frequencies to begin with. However, gradual
589 breakdown of barriers for gene flow can only explain changes observed between
590 years and not selection for introgressed alleles within a generation in males. Also,
591 allele frequency differences between sexes are not expected to be maintained without
592 selection over multiple generations. Second, a proportion of marker loci may have
593 been hitchhiking due to close genomic location with barrier loci and so missing from
594 males in 2004, but these hitchhikers are now becoming dissociated from the barrier
595 loci via recombination and consequently found in males in 2014. Again, this
596 hypothesis cannot explain the significant increase of introgressed alleles within a
597 generation in males. A third possibility is that the incompatibilities could be
598 environment-dependent, which could explain both within-generation and between-
599 years effect. Environment-dependence means that the negative effects of the
600 incompatibilities would be expressed only in certain environments. This situation is
601 comparable to genotype × genotype × environment interaction, where fitness effects
602 of a genetic variant are dependent on the genomic background it is found in, as well
603 as the external environment. Previous empirical evidence for environment-dependent
604 incompatibilities comes e.g. from yeast [33] and *Drosophila* [34]. Current results for
605 ants are most consistent with environment-dependent incompatibilities, but new

genome-wide data needs to be collected over multiple years and generations in order to rule out other hypotheses.

Can environment drive barrier leakage?

Environment dependent incompatibilities have been largely neglected in the speciation context: DMIs are assumed to be deleterious, no matter the environment. Yet, we know cases where barriers for gene flow hold in one environment but not in the other [14,33,34]. The fact that we see fluctuations rather than directional changes in the frequency of introgressed alleles at microsatellite loci (years 2004, 2008, 2011 and 2014) in the ant system is consistent with the idea of environment-dependent incompatibilities. What could be the selective agent? Temperature is one possible environmental variable that could affect the expression of incompatibilities due to two reasons. First, temperature differs between the current environments of the parental species, *F. aquilonia* being more Northern and *F. polyctena* being more Southern species. Second, temperature can show fluctuation between and within years. Indeed, the frequency of introgressed alleles in *F. aquilonia*–like males appears to co-vary with temperature during developmental season, but due to the limited time-series this correlation cannot be tested statistically (Figure 7). Further studies are needed to show if temperature is a key selective pressure that creates variation in the survival of ant hybrids. These analyses allow testing if incompatibilities are environment-dependent, an aspect that has been to a large extent ignored in speciation studies.

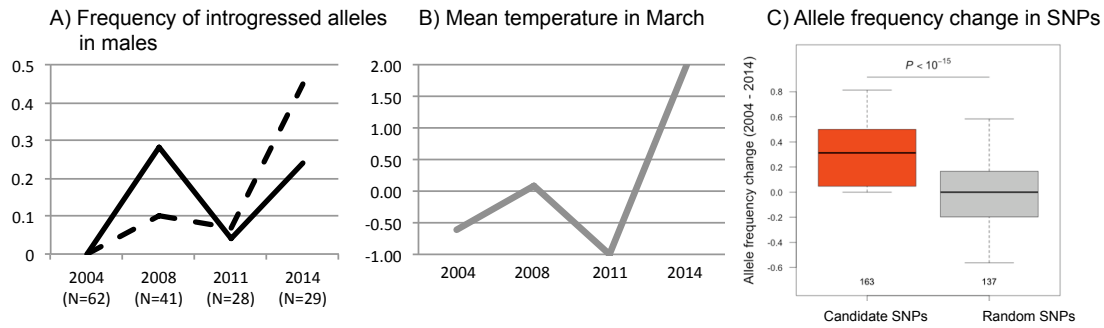


Figure 7. Frequency of introgressed alleles in adult *F. aquilonia*–like males appears to co-vary with yearly spring temperature. A) Frequency of alleles introgressed from *F. polyctena* to *F. aquilonia*–like males at two microsatellite loci (dashed FY13_190, straight FY15_222) in years 2004, 2008, 2011, 2014. B) Mean temperature (°C) in March during male development each year (Finnish Meteorological Institute, data from the nearest measurement point, Helsinki). C) Introgressed alleles at candidate SNPs (N=163) increase significantly more in frequency than alleles at random loci (N=137) between 2004 and 2014.

637 *Conclusions*
638 Here we bridged the gap between genome scan studies and fitness by mapping
639 candidate barrier loci between two recently diverged wood ant species and tested for
640 natural selection acting on candidate barrier regions. We do document selection at
641 candidate barrier loci, but it is acting on opposite direction than expected, favoring
642 introgression in males. This barrier leakage may depend on the environment and
643 fluctuate between years. These results highlight the dynamic nature of the ant system
644 that allows investigations into genomic and molecular consequences of hybridization,
645 areas where many questions still remain to be answered.
646
647 **Materials and Methods**
648
649 *Experimental design*
650 Our first aim was to use a genome scan approach to discover candidate barrier
651 regions, i.e. genomic regions that drive divergence between two wood ant species
652 despite of hybridization and gene flow. For this we used pooled whole genome
653 sequencing. Our second aim was to directly measure if natural selection is promoting
654 divergence and so speciation in the current-day hybrid population at the candidate
655 barrier regions using survival analysis and SNP genotyping.
656
657 *Discovery of candidate barrier regions with pooled genomic sequencing*
658 We used pooled genomic sequencing to compare male and female genomes between
659 *F. aquilonia*–like and *F. polyctena*–like hybrid lineages and to discover the candidate
660 barrier loci (i.e. genomic regions putatively associated to hybrid male breakdown).
661 We collected the samples used for pooled sequencing from the Långholmen hybrid
662 population (Kulmuni et al. 2010) in the year 2004. The samples were freshly frozen
663 and kept in -20°C and genomic DNA was extracted in the year 2010 from half a body
664 using Qiagen kit. We sequenced altogether four pools of individuals, where each pool
665 consisted of the following number of individuals; 1) 24 *F. aquilonia*–like males, 2) 24
666 *F. polyctena*–like males, 3) 24 *F. polyctena*–like females and 4) 24 *F. aquilonia*–like
667 females. The sample concentrations were checked with Qubit and pooled into the four
668 pools in equal amounts. Each pool was sequenced with 100bp paired-end sequencing
669 on its own lane in Illumina HiSeq2000 in the Institute for Molecular Medicine
670 Finland (FIMM). This resulted in 46,106,000 to 108,204,481 total number of reads

671 per pool. We quality trimmed reads by removing up to 20 bp that had phred score <
672 20 using FASTX-Toolkit. Next, we made denovo assemblies of each of the four
673 samples with Soapdenovo trying out different kmer sizes (31, 41, 51, 61, 71) for each
674 assembly. The *F. polyctena*–like male assembly with kmer size of 41 was best in
675 terms of completeness and quality (genome size: 222.6 Mb, 327480 contigs, average
676 contig length: 679 bp, N50 = 1748 bp) and chosen as our reference assembly. We then
677 mapped each sample back to the *F. polyctena*–like male reference assembly after
678 removing contigs of the assembly shorter than 500bp using Bowtie2 v2.0.2. Reads
679 mapped in proper pairs and with a mapping quality superior to 20 were filtered and
680 combined in a single mpileup file using samtools 1.4. Since coverage of the *F.*
681 *aquilonia*–like male pool was low (mean = 16, s.d. = 38), overlaps between read pairs
682 were kept. For all SNPs (see below) and in each pool, read counts were compared
683 with or without filtering of read pair overlaps using χ^2 tests. Over all pools, four SNPs
684 displayed significant allele frequency change ($P < 0.05$, for stringency no Bonferroni
685 correction was applied) and were removed from the dataset (Supplementary Figure 5).
686 The mpileup file was then converted in a synchronized file using Popoolation2 [35].
687 Indels and their 5-bp flanking sequences were masked, and only bi-allelic sites
688 displaying a minimum base quality of 20, coverage between 20 and 60 for each
689 population and a minor allele count of four across all populations were considered.
690 These steps led to the identification of 166,167 SNPs for which F_{ST} estimates were
691 computed from read count data using Popoolation2, adjusting for differences in
692 ploidy levels between haploid male and diploid female pools. These estimates were
693 compared with those obtained following a more recently developed approach [36]:
694 both methods provided similar results ($\rho = 0.96$) and identified a similar number of
695 SNPs differentially fixed in males (Popoolation2: 711; Poolfstat: 719, including all
696 the loci found using Popoolation2). Popoolation2 results were more conservative (i.e,
697 less differentially fixed SNPs) and were used for the rest of the study. For the
698 comparison between poolseq and SNP genotyping data sets, allele counts were
699 imputed from read counts using a maximum-likelihood approach [37], considering the
700 number of chromosomes sampled per pool.
701
702 *Gene Annotation, protein-protein interactions and GO term Enrichment Analysis*

703 We defined barrier loci as SNP positions where the male genomes (sampled in 2004)
704 from the *F. aquilonia*–like and *F. polyctena*–like lineages were fixed differently (i.e.
705 $F_{ST} = 1$, see Supplementary Figure 2), which led to identification of 711 SNPs. These
706 fall into to 610 assembled scaffolds. We created an automated pipeline to annotate
707 genomic regions around candidate barrier SNPs, taking advantage of the recent
708 release of the closely related *F. exsecta* genome [17]. First, using our assembly we
709 extracted a sequence of 5,000 bp centered on each of the 711 outlier SNPs. If the
710 contig was smaller than 5,000 bp, the full contig sequence was recovered. Since some
711 SNPs located on the same contig, and sometimes less than 5 kb apart, 639 unique
712 sequences were recovered and were blasted against the *F. exsecta* assembly. For each
713 queried sequence, hits covering less than 60% of its length were filtered out and the
714 best hit was kept per query based on e-value (over all queries, *e*-value $< 10^{-141}$). This
715 allowed to anchor 571 genomic regions on the *F. exsecta* assembly. The coordinates
716 of these regions were extended if needed to reach 5,000 bp, so that sampling effort
717 was equal among genomic regions. *F. exsecta* genes overlapping with best hits were
718 collected using the GenomeIntervals Bioconductor package v1.38. Overall, 590
719 unique genes overlapped with our 571 genomic regions. Sixty-three of the candidate
720 genomic regions did not contain any annotated gene. The mean number of genes per
721 candidate genomic region was 1.4, and the maximum was six. For each gene, its *F.*
722 *exsecta* transcript sequence was recovered and blasted against *D. melanogaster* r6.15
723 proteins after filtering for the longest protein per gene. Alignments below 150 bp and
724 with less than 35% identity were filtered out and the best hit was kept for each query
725 based on e-value (over all queries, *e*-value $< 10^{-20}$). This pipeline recovered 317 *D.*
726 *melanogaster* genes whose homologs reside in candidate barrier regions. These
727 candidate barrier genes were used for subsequent analyses (e.g., GO and PPI
728 enrichment analyses). For a complete list of scaffolds annotated with their gene names
729 see Supplementary Table 1. GO term enrichment analysis was performed using the
730 Gene Ontology Consortium tool and protein interactions were analyzed using
731 STRING database and *D. melanogaster* homologs for our candidate genes. We
732 performed 1,000 simulations to assess whether the observed PPI enrichment indicated
733 by STRING was significantly different from what would be expected for a random set
734 of SNPs from our dataset. For each simulation, we randomly drew 711 SNPs from the
735 total dataset (166,167 SNPs) and annotated their flanking regions using the same
736 automated pipeline and parameter values as presented above. The number of genes

737 recovered per simulation varied between 267 and 368 (mean = 319). For all
738 simulations and empirical data, PPI enrichment values were computed with STRING
739 v10 using the STRINGdb Bioconductor package [20], setting the score threshold to
740 800.
741
742 We investigated the location of the candidate barrier SNPs blasting to the genome of
743 closely related *F. exsecta*[17] (277 Mb, 14617 scaffolds, N50 = 997654), as our own
744 genomic assembly is highly fragmented.
745
746 *Testing for selection by genotyping candidate barrier and random SNPs*
747 To test for selection acting on candidate barrier SNPs we genotyped a set of candidate
748 and random SNPs from samples collected from the study population in 2014. These
749 samples were used to test if significant allele frequency changes occur between larval
750 and adult stages in candidate barrier SNPs but not in random SNPs, as expected if
751 these barrier SNPs mark genomic regions under selection. Genotyping was done at
752 individual level for a total of 196 individuals (Supplementary table 2). Samples were
753 randomly assigned to 96-well plates for genotyping.
754
755 Primer design and genotyping were done at LGC genomics using KASP genotyping
756 chemistry. We randomly chose 350 SNPs from the total SNP dataset. Out of these
757 180 were appropriate for primer design and genotyping. We further chose 183
758 candidate barrier SNPs out from the 711 SNPs identified previously. Candidate
759 barrier SNPs had F_{ST} of 1 between the male genomes but F_{ST} varied between females
760 at these loci. We aimed at variable F_{ST} estimates between females at the genotyped
761 barrier SNPs and initially chose the 50 SNPs with lowest F_{ST}, 50 SNPs with highest
762 F_{ST} and 100 randomly chosen from the remaining (Figure S1). Of these, 144 SNPs
763 were suitable for genotyping. Therefore, another 106 candidate barrier SNPs were
764 extracted, of which 76 passed the LGC primer design pipeline. In total 220 candidate
765 barrier SNPs were suitable for genotyping and from these we chose 183 SNPs
766 (Supplementary Figure 1). After removal of SNPs or individuals with more than 10%
767 missing data, diploid males from the *F. polyctena*–like lineage and three ambiguous
768 individuals (see Supplementary Figure 3), we were left with 181 individuals (for the
769 *F. polyctena*–like females, early stage: 27, late stage: 32, for the *F. aquilonia*–like

individuals, males early stage: 32, late stage: 32, females early stage: 31, late stage: 31) genotyped at 300 SNPs (137 random and 163 putative barrier loci).

Linkage disequilibrium could bias our results if putative barrier SNPs are non-independent or if putative random loci are actually located close to barrier regions. We took advantage of the more contiguous *F. exsecta* genome assembly and pruned the SNP genotyping data based on the location of BLAST hits in the *F. exsecta* genome (see above for BLAST parameters). Since the extent of linkage disequilibrium decay is unknown, we considered three different pruning intervals for SNPs which located on the same *F. exsecta* scaffolds: 50 kb (before pruning: $N_{barrier}$ = 163, N_{random} = 137; after pruning: $N_{barrier}$ = 137, N_{random} = 127), 100 kb ($N_{barrier}$ = 121, N_{random} = 122) and 500kb ($N_{barrier}$ = 80, N_{random} = 100). We then tested if this had impact on our estimates for F_{IS} in females and allele frequency change in males between early and late developmental stages by re-running analysis with the different pruned data sets.

Statistical analyses

We tested for significant differences in allele counts in *F. aquilonia*–like males between larval and adult stages at candidate barrier SNPs compared to the random SNPs using generalized linear mixed effects model in R [38]. We used locus type (candidate or barrier) and developmental stage (larva or adult) as explanatory variables and locus_ID as a random factor. F_{IS} values were estimated per genotyped locus and per group using the HierFstat package [39]. To test if F_{IS} estimates were significantly different at candidate barrier loci in females between larval and adult stages we used linear mixed effects model [38] and locus type, developmental stage and group (*F. polyctena* –like or *F. aquilonia*–like) as explanatory variables and locus_ID as a random factor.

References

1. Mayr E. Populations, species, and evolution; an abridgment of Animal species and evolution. Belknap Press of Harvard University Press; 1970.
2. Ravinet M, Faria R, Butlin RK, Galindo J, Bierne N, Rafajlović M, et al. Interpreting the genomic landscape of speciation: a road map for finding barriers to gene flow. J Evol Biol. 2017;30: 1450–1477. doi:10.1111/jeb.13047
3. Dobzhansky T. Studies on Hybrid Sterility. II. Localization of Sterility Factors

804 in Drosophila Pseudoobscura Hybrids. Genetics. 1936;21: 113–35.
805 4. Muller HJ. Isolating Mechanisms, Evolution, and Temperature. In: Biological
806 Symposia [Internet]. 1942 [cited 25 Sep 2018] pp. 71–125. Available:
807 https://www.scopus.com/record/display.uri?eid=2-s2.0-
808 84899736557&origin=inward
809 5. Kulmuni J, Westram AM. Intrinsic incompatibilities evolving as a by-product
810 of divergent ecological selection: Considering them in empirical studies on
811 divergence with gene flow. Mol Ecol. Wiley/Blackwell (10.1111); 2017;26:
812 3093–3103. doi:10.1111/mec.14147
813 6. Martin SH, Dasmahapatra KK, Nadeau NJ, Martin SH, Dasmahapatra KK,
814 Nadeau NJ, et al. Genome-wide evidence for speciation with gene flow in
815 Heliconius butterflies Genome-wide evidence for speciation with gene flow in
816 Heliconius butterflies. 2013; 1817–1828. doi:10.1101/gr.159426.113
817 7. Soria-Carrasco V, Gompert Z, Comeault A a, Farkas TE, Parchman TL,
818 Johnston JS, et al. Stick insect genomes reveal natural selection's role in
819 parallel speciation. Science. 2014;344: 738–42. doi:10.1126/science.1252136
820 8. Cruickshank TE, Hahn MW. Reanalysis suggests that genomic islands of
821 speciation are due to reduced diversity, not reduced gene flow. Mol Ecol.
822 Wiley/Blackwell (10.1111); 2014;23: 3133–3157. doi:10.1111/mec.12796
823 9. Wolf JBW, Ellegren H. Making sense of genomic islands of differentiation in
824 light of speciation. Nat Rev Genet. Nature Publishing Group; 2017;18: 87–100.
825 doi:10.1038/nrg.2016.133
826 10. Goropashnaya A V., Fedorov VB, Pamilo P. Recent speciation in the Formica
827 rufa group ants (Hymenoptera, Formicidae): inference from mitochondrial
828 DNA phylogeny. Mol Phylogenet Evol. 2004;32: 198–206.
829 doi:10.1016/j.ympev.2003.11.016
830 11. Kulmuni J, Seifert B, Pamilo P. Segregation distortion causes large-scale
831 differences between male and female genomes in hybrid ants. Proc Natl Acad
832 Sci U S A. 2010;107: 7371–7376.
833 12. Kulmuni J, Pamilo P. Introgression in hybrid ants is favored in females but
834 selected against in males. Proc Natl Acad Sci U S A. 2014;111: 1323045111-.
835 doi:10.1073/pnas.1323045111
836 13. Ghenu A-H, Blanckaert A, Butlin RK, Kulmuni J, Bank C. Conflict between
837 heterozygote advantage and hybrid incompatibility in haplodiploids (and sex

838 chromosomes). Mol Ecol. 2018; doi:10.1111/mec.14482
839 14. Schumer M, Powell DL, Delclós PJ, Squire M, Cui R, Andolfatto P.
840 Assortative mating and persistent reproductive isolation in hybrids. 2017;
841 doi:10.1073/pnas.1711238114
842 15. Beresford J, Elias M, Pluckrose L, Sundström L, Butlin RK, Pamilo P, et al.
843 Widespread hybridization within mound-building wood ants in Southern
844 Finland results in cytonuclear mismatches and potential for sex-specific hybrid
845 breakdown. Mol Ecol. Wiley/Blackwell (10.1111); 2017;26: 4013–4026.
846 doi:10.1111/mec.14183
847 16. Helms Cahan S, Keller L. Complex hybrid origin of genetic caste
848 determination in harvester ants. Nature. 2003;424: 306–309.
849 doi:10.1038/nature01744
850 17. Dhaygude K, Nair A, Johansson H, Wurm Y, Sundström L. The first draft
851 genomes of the ant Formica exsecta, and its Wolbachia endosymbiont reveal
852 extensive gene transfer from endosymbiont to host. bioRxiv. Cold Spring
853 Harbor Laboratory; 2018; 436691. doi:10.1101/436691
854 18. Goropashnaya A V., Fedorov VB, Seifert B, Pamilo P. Phylogenetic
855 relationships of Palaearctic Formica species (hymenoptera, Formicidae) based
856 on mitochondrial cytochrome b sequences. PLoS One. 2012;7.
857 doi:10.1371/journal.pone.0041697
858 19. ROSENGREN M, ROSENGREN R, SÖDERLUND V. Chromosome numbers
859 in the genus Formica with special reference to the taxonomical position of
860 Formica uralensis Ruzsk. and Formica truncorum Fabr. Hereditas. John Wiley
861 & Sons, Ltd (10.1111); 1980;92: 321–325. doi:10.1111/j.1601-
862 5223.1980.tb01715.x
863 20. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J,
864 et al. STRING v10: protein–protein interaction networks, integrated over the
865 tree of life. Nucleic Acids Res. Oxford University Press; 2015;43: D447–D452.
866 doi:10.1093/nar/gku1003
867 21. Rellstab C, Zoller S, Tedder A, Gugerli F, Fischer MC. Validation of SNP
868 Allele Frequencies Determined by Pooled Next-Generation Sequencing in
869 Natural Populations of a Non-Model Plant Species. Marais GA, editor. PLoS
870 One. 2013;8: e80422. doi:10.1371/journal.pone.0080422
871 22. Byers KJRP, Xu S, Schlüter PM. Molecular mechanisms of adaptation and

872 speciation: why do we need an integrative approach? Mol Ecol. 2017;26: 277–
873 290. doi:10.1111/mec.13678
874 23. Turner LM, White M a., Tautz D, Payseur B a. Genomic Networks of Hybrid
875 Sterility. PLoS Genet. 2014;10: 18–22. doi:10.1371/journal.pgen.1004162
876 24. Lindtke D, Buerkle CA. The genetic architecture of hybrid incompatibilities
877 and their effect on barriers to introgression in secondary contact. Evolution (N
878 Y). 2015; n/a-n/a. doi:10.1111/evo.12725
879 25. Porter AH, Johnson NA. Speciation despite Gene Flow When Developmental
880 Pathways Evolve Published by : Society for the Study of Evolution Stable
881 URL : http://www.jstor.org/stable/3094654. Society. 2010;56: 2103–2111.
882 26. Luisi P, Alvarez-Ponce D, Pybus M, Fares MA, Bertranpetit J, Laayouni H.
883 Recent positive selection has acted on genes encoding proteins with more
884 interactions within the whole human interactome. Genome Biol Evol. 2015;7:
885 1141–1154. doi:10.1093/gbe/evv055
886 27. Coyne JA, Orr HA. Speciation. Sinauer Associates; 2004.
887 28. Rogers SM, Bernatchez L. The genetic basis of intrinsic and extrinsic post-
888 zygotic reproductive isolation jointly promoting speciation in the lake whitefish
889 species complex (Coregonus clupeaformis). J Evol Biol. 2006;19: 1979–1994.
890 doi:10.1111/j.1420-9101.2006.01150.x
891 29. Nei M. The new mutation theory of phenotypic evolution. Proc Natl Acad Sci
892 U S A. National Academy of Sciences; 2007;104: 12235–42.
893 doi:10.1073/pnas.0703349104
894 30. Stockan JA, Robinson EJH, Trager JC, Yao I, Seifert B. Introducing wood
895 ants: evolution, phylogeny, identification and distribution. In: Stockan JA,
896 Robinson EJH, editors. Wood Ant Ecology and Conservation. Cambridge:
897 Cambridge University Press; 2016. pp. 1–36.
898 doi:10.1017/CBO9781107261402.002
899 31. True JR, Haag ES. Developmental system drift and flexibility in evolutionary
900 trajectories. Evol Dev. 3: 109–19.
901 32. Nosil P, Villoutreix R, Carvalho CF De, Farkas TE, Soria-carrasco V, Feder
902 JL, et al. Natural selection and the predictability of evolution in Timema stick
903 insects. 2018;770: 765–770.
904 33. Hou J, Friedrich A, Gounot J-S, Schacherer J. Comprehensive survey of
905 condition-specific reproductive isolation reveals genetic incompatibility in

yeast. Nat Commun. Nature Publishing Group; 2015;6: 7214. doi:10.1038/ncomms8214

34. Hoekstra LA, Siddiq MA, Montooth KL. Pleiotropic effects of a mitochondrial-nuclear incompatibility depend upon the accelerating effect of temperature in Drosophila. Genetics. Genetics Society of America; 2013;195: 1129–39. doi:10.1534/genetics.113.154914

35. Kofler R, Pandey RV, Schlötterer C. PoPoolation2: Identifying differentiation between populations using sequencing of pooled DNA samples (Pool-Seq). Bioinformatics. 2011;27: 3435–3436. doi:10.1093/bioinformatics/btr589

36. Hivert V, Leblois R, Petit EJ, Gautier M, Vitalis R. Measuring Genetic Differentiation from Pool-seq Data. Genetics. Genetics; 2018;210: 315–330. doi:10.1534/genetics.118.300900

37. Leblois R, Gautier M, Rohfritsch A, Foucaud J, Burban C, Galan M, et al. Deciphering the demographic history of allochronic differentiation in the pine processionary moth *Thaumetopoea pityocampa*. Mol Ecol. Wiley/Blackwell (10.1111); 2018;27: 264–278. doi:10.1111/mec.14411

38. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using **lme4**. J Stat Softw. 2015;67: 1–48. doi:10.18637/jss.v067.i01

39. GOUDET J. hierfstat, a package for r to compute and test hierarchical F-statistics. Mol Ecol Notes. John Wiley & Sons, Ltd (10.1111); 2005;5: 184–186. doi:10.1111/j.1471-8286.2004.00828.x

Acknowledgements

We thank Pekka Pamilo and Simon H. Martin for their valuable comments on the manuscript, Petri Kemppainen for discussions and CSC – IT Center for Science, Finland, for computational resources. JK was supported by Human Frontier Science Program Long-term fellowship and Academy of Finland grant nro. 309580. JK and RB designed the study. JK, PN, LP, IS and KD analyzed the data. JK, PN, LP, IS and RB wrote the manuscript. Data will be deposited into Dryad upon acceptance.