Hybridizing wood ants allow testing for natural selection at candidate barrier loci underlying speciation

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

The current aim of speciation research is to pin-point which genomic regions serve as barriers for gene flow and drive divergence during speciation. At the barrier loci, natural selection is assumed to act against gene flow. Many current approaches, however, rely on indirect measures of gene flow and natural selection. Here we present a system to test the action of natural selection at candidate barrier loci in a natural population. We utilize haplodiploidy to identify candidate barrier loci between two wood ant species and combine survival analysis with SNP genotyping to test for natural selection acting at candidate barrier loci. We find multiple candidate loci distributed over a large part of the genome displaying signatures of natural selection. Surprisingly, however, we find that a proportion of the barrier loci show leakage between data sets collected in 2004 and 2014. We also show that, on average selection favored introgression at candidate barrier loci in year 2014. We discuss reasons for barrier leakage, including environment-dependent selection, formation of compatible combinations of parental alleles and recombination breaking associations between causal and hitchhiking loci. Integrating data on survival allows us to move

beyond genome scan studies, bringing additional evidence for natural selection acting in genomic islands of divergence.

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

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[2,3]. Barrier loci could contribute to reproductive isolation by allowing differential adaptation between diverging lineages[4]. Alternatively, barrier loci could consist of genes incompatible between the diverging lineages (Dobzhansky-Muller incompatibilities[5,6], DMIs) driven by drift or divergent selection[7].

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[8–11]). The underlying assumption in these genomic 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[12–14]. The main problem lies in the fact that several other evolutionary forces (such as low recombination rate or drift[10,15]) can bring about genomic regions of seemingly high differentiation between populations. Thus, we need more studies that demonstrate the action of divergent natural selection in 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[16]. 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[17,18].

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[18]. 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. 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[19]. 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[20]; 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)[21]. Both lineages are of hybrid origin and 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. 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 were estimated to die[18]. Selection is weaker on the males of the *F. aquilonia*–like lineage, as is the evidence for selection favoring female heterozygotes.

Here we discover and annotate candidate barrier loci, i.e. the genomic regions that associate with hybrid male breakdown and thus act as barriers for gene flow between the two wood ant species. We then test if natural selection is acting on these candidate barrier regions in nature using SNP genotyping and survival analysis. This allows for testing the role of natural selection in genomic islands of divergence and reproductive isolation within a natural population, which has not been possible in most of the genomic studies of speciation.

Results

Multiple barrier loci throughout the genome are candidates for hybrid male breakdown

We used pooled genomic sequencing to discover the genomic regions associated with hybrid male breakdown (see Figure 1 for experimental design). We sequenced four samples, each consisting of 24 individuals: *F. polyctena*–like males, *F. polyctena*–like females (unmated queens), *F. aquilonia*–like males and *F. aquilonia* –like females (unmated queens). These individuals were classified into lineages based on six to seven diagnostic microsatellite alleles[17]. Due to haplodiploidy, this resulted in 24 and 48 chromosomes sampled for male and female pools, respectively. These pools represent population samples and came from two to four nests each, all of which have hundreds of reproductive queens and relatedness approaching zero. We sequenced each pool with 100 bp paired-end reads on its own lane in Illumina HiSeq2000 and made a *de novo* assembly from each pool. Subsequently, the assembly of *F. polyctena*–like males was used as a reference (genome size = 222.6 Mb, N50 = 1748 bp, 327,480 total scaffolds). Each pool was mapped to the reference assembly and SNPs were called after filtering. We retained 166,167 bi-allelic SNPs displaying coverage between 20 and 60 in each pool and minor allele count of four over all pools.

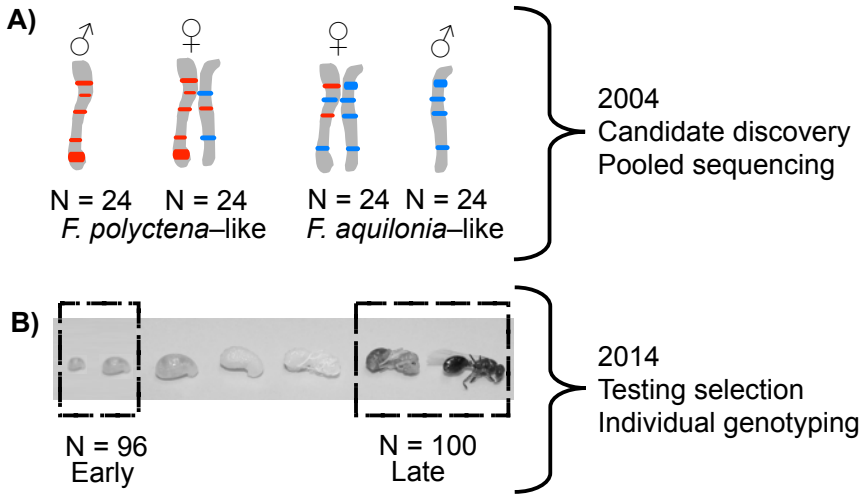

Figure 1. Experimental design for A) Discovering candidate barrier regions with pooled genomic sequencing (by sampling adults), with a schematic representation of the male (haploid) and the female (diploid) chromosomes of the two lineages. Red rectangles represent alleles originating from the *F. polyctena*-like lineage and blue represent alleles originating from *F. aquilonia*-like lineage. B) Testing signs of selection between larval stage (Early) and adult stage (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} 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 males of the *F. aquilonia*–like and *F. polyctena*–like lineages, but no fixed differences between the female pools. Mean F_{ST} in females for the SNPs fixed differently in males was 0.34 (s.e. = 5.83×10^{-3}, Supplementary Figure 1). The alternatively fixed SNPs between the male pools represent genomic regions of high differentiation between the two lineages despite their shared hybrid history, and thus mark candidate barrier loci that are predicted to contribute to reproductive isolation and speciation. These 711 SNPs fell into 610 assembled contigs (cumulative size: 2.09 Mb, 0.94% of the genome), whose lengths vary between 515 – 23,815 bp (mean = 3430 bp). Seventy-eight contigs had more than two barrier SNPs (maximal number of barrier SNPs on a single contig was 5).

We were interested in how these candidate barrier loci locate in the genome, because this can provide information about the origin of reproductive isolation and play a role in how efficient the barrier loci are in maintaining divergence despite hybridization and gene flow. If candidate barrier loci are co-localized in the genome their origin and maintenance could be explained by selection on a few genomic regions that could, for example, harbor structural re-arrangements. Alternatively, candidate barrier loci scattered across the genome could effectively reduce gene flow over much of the

192 genome. We used the genome of the closely-related *Formica exsecta*[22] (277 Mb,
193 14,617 scaffolds, N50 = 997,654), which is better assembled compared to the poolseq
194 genome assembly, to map the location of candidate barrier loci. *F. exsecta* is
195 estimated to have diverged from the parental species of our hybrid population 5
196 Mya[16]. We used the 610 contigs containing candidate barrier SNPs as queries and
197 searched the *F. exsecta* genome assembly using BlastN. If our query scaffold was
198 larger than 5 000 bp we used +/- 2 500 bp surrounding the candidate barrier SNP as
199 query. Our candidate barrier loci map to a total of 134 *F. exsecta* contigs, with a
200 cumulative coverage of 56.2 % of the *F. exsecta* genome, both of which are
201 significantly less than for a similar number of random SNPs in our data set
202 (Supplementary Figure 2). Haploid chromosome number varies between 26 and 28 in
203 *Formica*[23]. Assuming evenly-sized chromosomes our candidate barrier loci are likely
204 to be situated in over 10 chromosomes.
205
206 *Candidate barrier loci form a network of interacting proteins*
207 Next, we were interested in the possible functions of our candidate barrier loci, as
208 they can be informative about the putative selection pressures and mechanisms of
209 hybrid male breakdown. We annotated each of the 610 contigs containing candidate
210 barrier SNPs, first extracting the transcripts of the genes located in those regions from
211 the closely related *F. exsecta*. Out of 571 candidate barrier regions (see Material and
212 Methods; each region was defined as 5000 bp around the candidate barrier SNP) that
213 we were able to anchor in *F. exsecta* (all blastn *e*-values < 10^{-141}), we recovered 590
214 unique genes. Sixty-three of the candidate genomic regions did not contain any
215 annotated gene. The mean number of genes per candidate genomic region was 1.4,
216 and the maximum was six. For each gene, its *F. exsecta* transcript sequence was
217 recovered and blasted (blastx) against *Drosophila melanogaster* r6.15 proteins
218 recovering 317 genes, whose homologs reside in candidate barrier regions
219 (Supplementary Table 1).
220
221 The genetic model for hybrid breakdown suggests inviability and sterility are caused
222 by dysfunction and incompatibilities between diverged genes from the parental
223 species. We thus asked if there was any evidence for protein-protein interactions
224 between the gene products in candidate barrier regions using the recovered *D.*
225 *melanogaster* proteins and the STRING database[24], which contains information on

known and predicted protein-protein interactions. We found that candidate barrier regions harbor proteins that show significantly more interactions with each other than a random set of proteins drawn from the *D. melanogaster* genome (PPI enrichment *p*-value = 9.65×10^{-13}, Figure 2). Out of 268 genes annotated and having information in the STRING database, 167 genes fall into a single interaction network, with evidence for protein-protein interactions in *D. melanogaster* or other species. Seventy-four of the network genes had evidence for interactions which were experimentally determined or obtained from curated database (PPI enrichment *p*-value = 0.03). However, randomly drawing 711 SNPs from our pooled sequencing data 1000 times showed that similar enrichment in protein-protein interactions (i.e. network) could be retrieved for a random set of SNPs as well. This suggests either a bias towards functional and interacting sets of genes in our total SNP dataset or a bias arising due to conservation (only genes conserved between ants and *D. melanogaster* can be included in the analysis). 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.

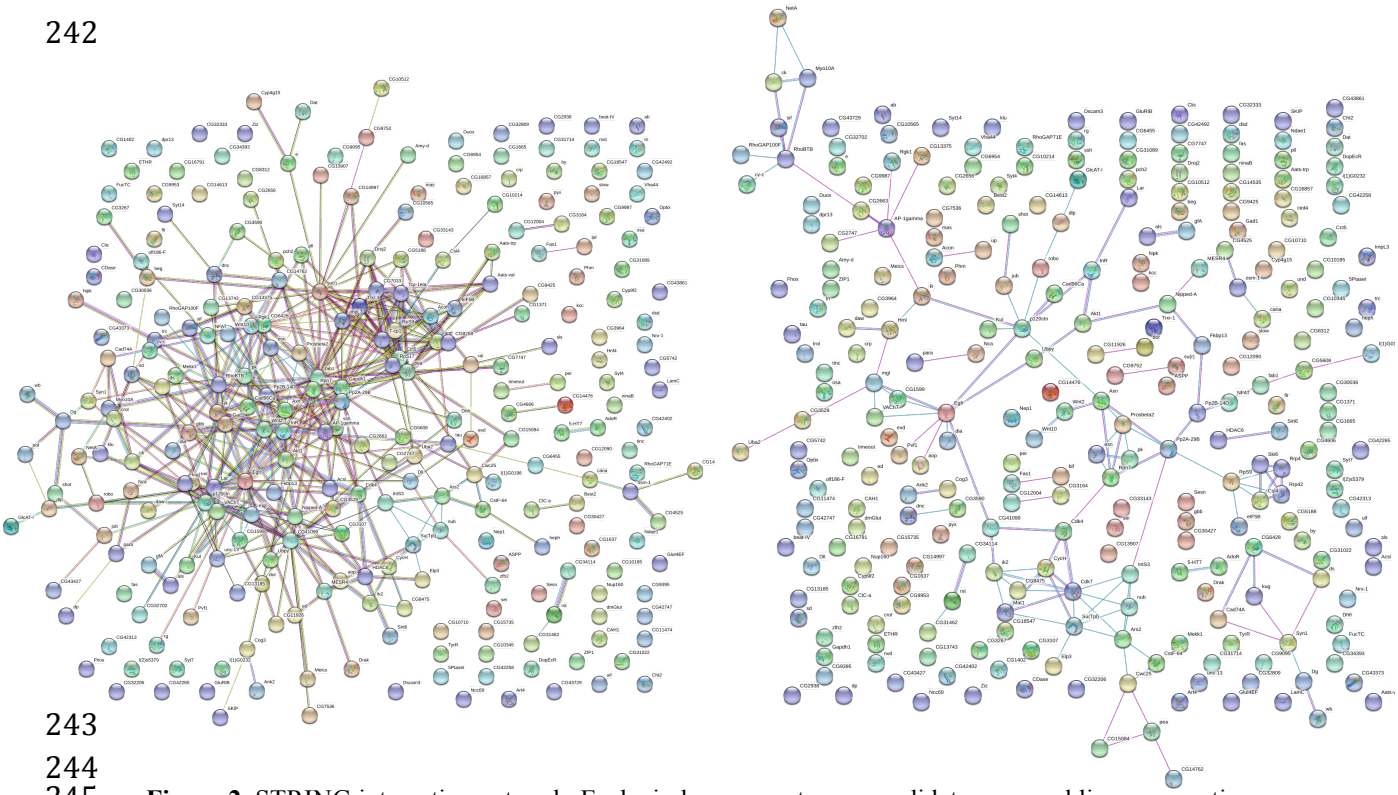

Figure 2. STRING interaction network. Each circle represents one candidate gene and lines connecting them indicate support for interaction between the genes. Majority of interactions fall into a single network. Line color indicates following: blue = data from curated databases, pink = experimentally 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.

251

252 Next, we were interested in the functions of genes within candidate barrier regions.
253 For this we used the *D. melanogaster* gene IDs. The top three biological processes
254 characterizing our candidate barrier genes are "developmental process" ($p = 3.5\times10^{-}$
255 ^{18}), "system development" ($p = 3.5\times10^{-18}$), and "single-organism developmental
256 process" ($p = 4.03\times10^{-18}$). Similarly, the top three molecular functions are "ion
257 binding" ($p = 8.55\times10^{-6}$), "protein binding" ($p = 3.67\times10^{-5}$) and "calcium ion binding"
258 ($p = 8.74\times10^{-5}$). These biological processes and molecular functions were highly
259 significantly enriched when compared to the genome average of *Drosophila*
260 *melanogaster*. However, they were not significantly enriched if compared to a random
261 set of SNPs drawn from our data, as all of them are found in over 5 % of our
262 simulations.
263

264 *Testing for signs of selection at candidate barrier loci in a natural population*
265 Next we tested if the newly annotated candidate barrier regions experienced selection
266 during development as expected based on our previous results. We expected
267 introgressed alleles in barrier regions to decline in frequency across different life-
268 stages in the males, whereas in females they would be favoured in heterozygote state
269 as shown in our previous marker-based study[18]. We tested for selection during
270 development by genotyping altogether 96 individuals at early (larva) and 100
271 individuals at late (adult or late stage pupa) developmental stages (Supplementary
272 Table 2), at 180 random and 183 candidate barrier SNPs. Out of these, 163 candidate
273 barrier SNPs and 137 random SNPs were successfully genotyped. The 137 random
274 SNPs were located on 136 contigs (cumulative size: 484 kb, 0.22% of the genome),
275 whereas the 163 candidate barrier SNPs located on 159 contigs within our assembly
276 (cumulative size: 523 kb, 0.23% of the genome). Individuals used in genotyping were
277 collected in Spring 2014 from the same hybrid population as the one sampled for the
278 pooled sequencing analysis ten years earlier.
279

280 The SNP genotyping confirmed that within the hybrid population, *F. polyctena*–like
281 and *F. aquilonia*–like lineages are genetically distinct from one another
282 (Supplementary Figure 3). We found two small female larvae, which were genetically
283 intermediate and had unique combinations of putative parental alleles from the two

284 lineages suggesting they were early-generation hybrids between the lineages
285 (Supplementary Figure 3). The *F. polyctena*–like lineage was previously shown to
286 experience stronger selection than the *F. aquilonia*–like lineage. Especially, over 90
287 % of the male eggs laid in the *F. polyctena*–like lineage were estimated to be inviable
288 due to incompatible combinations of introgressed and putative ancestral alleles at
289 microsatellite loci[18]. Unfortunately, we were able to sample only 8 males from this
290 lineage, five of which turned out to be diploid, thus *F. polyctena*–like males were
291 excluded from the analysis. The following analyses were performed for *F. polyctena*-
292 like females, *F. aquilonia*-like males and *F. aquilonia*-like females.
293
294 First we asked if adult allele frequencies inferred from the pooled sequencing
295 correlated with adult allele frequencies estimated from the SNP genotyping data, as
296 the two data sets have been collected ten years apart and allele frequencies were
297 estimated in different ways. Over all the genotyped SNPs, allele frequencies between
298 the two data sets correlated well in *F. polyctena*–like females (Spearman's
299 correlation, $\rho = 0.87$, $CI_{95} = [0.82, 0.90]$, $p < 0.001$) and in *F. aquilonia*–like females
300 ($\rho = 0.91$, $CI_{95} = [0.88, 0.94]$, $p < 0.001$) (Figure 3). The correlation was lower for the
301 males with a Spearman's correlation coefficient of 0.71 ($CI_{95} = [0.64, 0.76]$, $p <$
302 0.001) between adult allele frequencies estimated from pooled sequencing and
303 genotyping (Figure 3). The lower correlation in males compared to females can be
304 explained, at least in part, by the lower number of sampled chromosomes for
305 (haploid) males compared to (diploid) females creating a larger error in the estimated
306 allele frequencies. Overall, correlations between allele frequency estimates are in line
307 with other studies focusing on the accuracy of allele frequency estimates derived from
308 poolseq[25,26] and suggest pooled sequencing and SNP genotyping gave similar results.
309

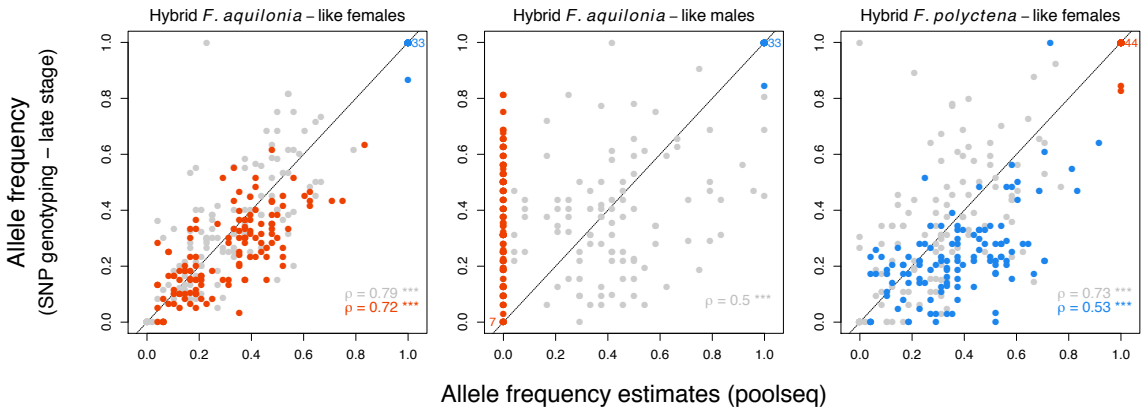

Figure 3. Allele frequencies correlate between pooled sequencing (adults sampled in 2004) and SNP genotyping in late stage (adults sampled in 2014). Putative barrier loci are colored according to their polarization (red: allele introgressed from *F. polyctena*-like to *F. aquilonia*-like, blue: allele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

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

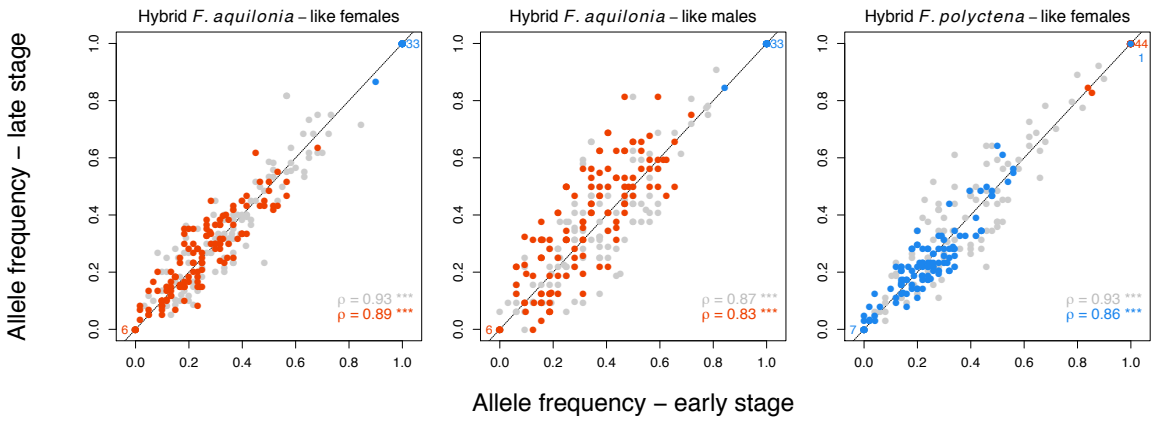

Figure 4. Allele frequency correlation between early (larva) and late (adult) developmental stages in random (grey) and candidate barrier loci (red: allele introgressed from *F. polyctena*-like to *F. aquilonia* -like, blue: allele introgressed from *F. aquilonia*-like to *F. polyctena*-like) in year 2014. No strong allele frequency change was observed from larva to adult in females. 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 (***: $p < 0.001$).

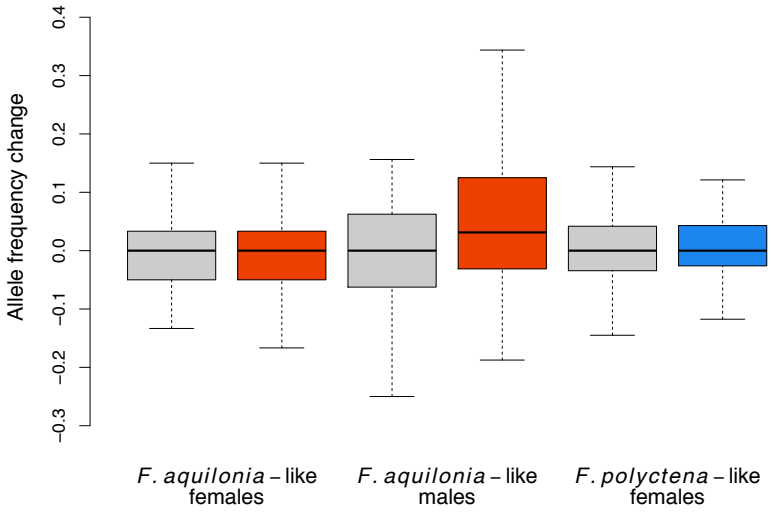

Figure 5. Mean allele frequency change between early (larva) and late (adult) developmental stages in random (grey) and candidate barrier loci (red or blue) in year 2014. Allele frequencies at barrier loci are polarized according to the introgressed allele. Candidate barrier SNPs have significantly stronger 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 (Table 1). Indeed, there was a tendency for more negative mean F_{IS} values (i.e. greater excess of heterozygosity) in candidate barrier loci at the late stage compared to the early stage in both female lineages (Figure 6), but this tendency was not statistically significant in either lineage (*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 at both larval (z-value = –4.404, $p < 0.001$) and adult (z-value= –7.133, $p < 0.001$) stages in *F. polyctena*–like females. This result is a signature of either (i) strong allele frequency differences between parental genotypes or (ii) selection for heterozygosity that has already acted between egg and larval stages at candidate barrier loci.

Table 1. Mean F_{IS} estimates in genotyped SNPs in females

	Random loci	Candidate barrier loci
F. aquilonia–like females		
Early stage	-0.085	-0.085
Late stage	-0.098	-0.116
F. polyctena–like females		
Early stage	-0.073	-0.179
Late stage	-0.031	-0.200

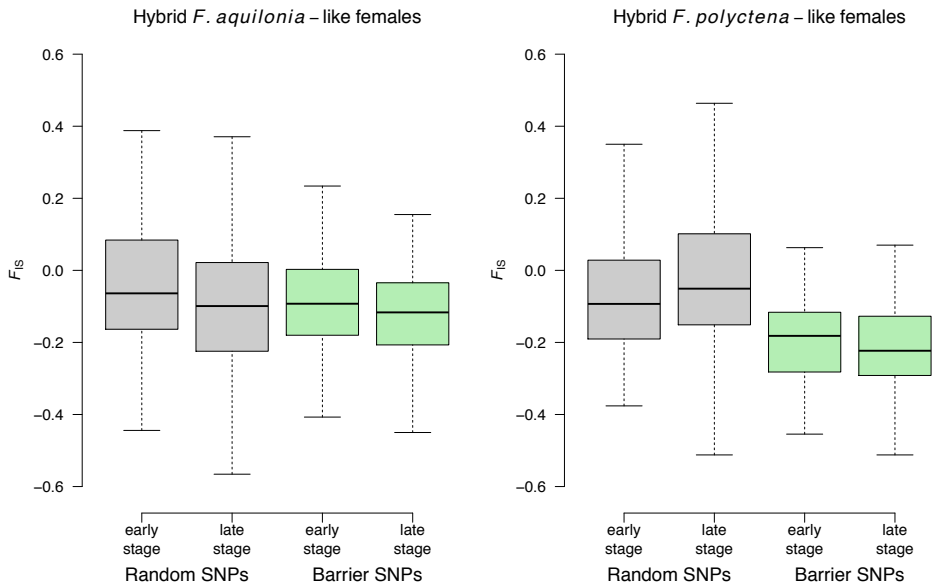

Figure 6. Mean F_{IS} estimates in early (larva) and late (adult) developmental stages in random (grey) and candidate barrier loci (green) in year 2014.

Then, we tested for allele frequency changes in *F. aquilonia*–like males, expecting the introgressed allele to decrease in frequency during development. Allele frequencies were significantly different between larval and adult developmental stages in candidate barrier SNPs compared to the random SNPs (glmer, *z*-value= –2.98, p = 0.00286), the candidate barrier SNPs showing more frequency change during development (Figure 5). However, the frequencies of the introgressed alleles increased from larval to adult developmental stages in *F. aquilonia*–like males (Figure 4), a pattern opposite to our expectations. Significant increase of introgressed alleles at candidate barrier loci in contrast to alleles at random loci suggests selection favored the introgressed alleles in *F. aquilonia*–like males during development. Unfortunately, we were unable to test selection acting at candidate barrier loci in *F. polyctena*–like males, the lineage where we had the strongest expectation of negative

380 selection, because too few *F. polyctena*–like males were sampled from the population.
381 The low occurrence of these males is consistent with their estimated high mortality[18].
382
383 *Significant variation in the strength of barriers through time*
384 Introgressed (i.e., *F. polyctena*–like) alleles were absent from adult *F. aquilonia*–like
385 males at 163 candidate barrier loci in year 2004 (allele frequency of zero in the
386 poolseq data). However, in year 2014 significant proportion of these loci harbored
387 alleles introgressed from the *F. polyctena*–like lineage (123 loci, i.e. 75.5%) to *F.*
388 *aquilonia*–like males. The mean frequency of introgressed alleles in these males was
389 0.36, the most common introgressed allele being at a frequency of 0.81. We observed
390 no population substructure within the *F. aquilonia*–like lineage (Supplementary
391 Figures 3 and 4). Accordingly, sampling different sub-populations within a lineage at
392 different years is unlikely to drive increase of introgressed allele frequencies between
393 2004 and 2014 in our samples of *F. aquilonia*–like males. Increase of introgressed
394 alleles in males is not likely to be caused by errors in male poolseq-based allele
395 frequency estimates either. All but one of the candidate barrier SNPs (33 out of 34
396 SNPs) that were initially fixed in both adult males and females of the *F. aquilonia*–
397 like lineage in the pooled sequencing data (year 2004) were still fixed in the
398 genotyping data (year 2014), showing consistency across data sets. If differences
399 between allele frequency estimates from 2004 and 2014 were caused by errors in
400 allele poolseq allele frequency estimates, this should affect all SNP loci equally.
401 However, this is not the case, as only candidate barrier SNPs that were polymorphic
402 in females of the *F. aquilonia*–like lineage have now "leaked" into *F. aquilonia*–like
403 males. Our results thus suggest that in year 2014 a proportion of the barrier loci are
404 "leaking" in *F. aquilonia*–like males. At these loci introgressed alleles were on
405 average selected for in males as evidenced by significant frequency increase between
406 larval and adult stages (see above), a pattern opposite to previous observations[17,18].
407
408 Using microsatellite genotyping, we further verified that increasing frequencies of
409 introgressed alleles at candidate barrier SNPs in *F. aquilonia*–like males did not
410 represent technical artifacts. Specifically, we genotyped the individuals collected in
411 2014 and used in SNP genotyping with nine previously used microsatellite markers[7,18]
412 and compared them to samples collected in 2004, 2008 and 2011 (Supplementary
413 Table 3). Microsatellite allele frequencies from 2014 paralleled the pattern observed

in candidate barrier SNPs in the same year. Indeed, *F. aquilonia*–like males in 2014 harbor alleles introgressed from the *F. polyctena*–like lineage that were missing from them in 2004 (Table 2). Specifically, the frequencies of two microsatellite alleles ($Fy3_{190}$ and $Fy15_{222}$) introgressed from *F. polyctena* to *F. aquilonia*–like males have fluctuated over the years (Table 2). Similar leakage of alleles introgressed from *F. aquilonia* to the *F. polyctena*–like males is not observed as the five introgressed microsatellite alleles have remained at a frequency of zero in adult males from 2004 (N=35) to 2008 (N=41) and 2011 (N=23).

Table 2. Frequencies of introgressed alleles (background shaded grey in males) at microsatellite loci in years 1996, 2004, 2008, 2011 and 2014. Data for years 1996-2011 from ref[18].

	Year	1996	1996	2004	2004	2004	2004	2008	2008	2008	2011	2011	2011	2014	2014	2014
Locus	**Allele**	**F. aquil.** ♀worker	**F. polyc.** ♀worker	**F. aquil.** ♂	**F. aquil.** ♀	**F. polyc.** ♂	**F. polyc.** ♀	**F. aquil.** ♂	**F. aquil.** ♀	**F. polyc.** ♂	**F. aquil.** ♂	**F. polyc.** ♂	**F. polyc.** ♀	**F. aquil.** ♂	**F. aquil.** ♀	**F. polyc.** ♀
Alleles introgressed from *F. aquilonia* -like lineage to *F. polyctena* -like lineage																
FE13	198	0,66	0,83	0,48	0,65	0,00	0,50	0,54	0,68	0,00	0,61	0,00	0,31	0,759	0,661	0,281
FE17	110	0,41	0,06	0,18	0,42	0,00	0,28	0,24	0,39	0,00	0,25	0,00	0,10	0,483	0,339	0,078
FE7	68	0,58	0,06	0,80	0,67	0,00	0,23	0,63	0,73	0,00	0,78	0,00	0,06	0,724	0,661	0,109
FL29	188	0,39	0,22	0,30	0,42	0,00	0,16	0,63	0,28	0,00	0,43	0,00	0,21	0,690	0,452	0,109
FY15	232	0,39	0,11	0,29	0,38	0,00	0,33	0,33	0,42	0,00	0,30	0,00	0,10	0,379	0,355	0,219
Alleles introgressed from *F. polyctena* -like lineage to *F. aquilonia* -like lineage																
FY15	222	0,12	0,17	0,00	0,07	0,50	0,50	0,28	0,11	0,44	0,04	0,57	0,52	0,241	0,113	0,422
FY3	190	0,22	0,56	0,00	0,18	0,20	0,42	0,10	0,19	0,10	0,07	0,13	0,49	0,448	0,129	0,500

Discussion

Several recent genome scan studies have used next generation sequencing to map genomic regions putatively under divergent natural selection and underlying reproductive isolation, e.g.[9,27–29]. Approaches can often be based solely on comparing genomes, because observing natural selection, collecting data on fitness or molecular characterization of loci underlying reproductive isolation is challenging. However, several suggestions have been made to move beyond genome scans with an integrative approach[4,30]. To bridge the gap between genotype and fitness in genome scan studies we first took advantage of male haploidy in ants and discovered genomic regions of high divergence (i.e. candidate barrier regions). We then tested if natural selection is acting on these genomic regions in a natural population using a SNP panel and survival analysis, expecting haploid males to show the strongest signals of selection against gene flow at barrier loci. Indeed, we find evidence of natural selection acting in the candidate barrier regions: in spring 2014 barrier SNPs showed significant frequency change in *F. aquilonia*–like males during development.

449 Surprisingly, selection acts in a direction opposite to our expectation, favoring
450 introgression in males. Thus, the ant system is dynamic and not likely to be in an
451 equilibrium state, as at least a proportion of the barriers showed leakage in 2014
452 compared to 2004.
453
454 *Considering barrier loci that evolve in a network*
455 Hybrid breakdown is expected to result from deleterious epistatic interactions (i.e.
456 intrinsic incompatibilities) between diverged genes of the parental species [5,6].
457 However, two fundamental questions about incompatibilities remain to be answered.
458 First, is hybrid breakdown based on few or many loci? Second, if breakdown is based
459 on many loci do these represent multiple two-locus interactions or do incompatibility
460 loci interact in networks or pathways? Our results provide insights into both of these
461 questions. We found that candidate barrier and hitchhiking loci likely cover a large
462 proportion of the genome in two *Formica* ant species, which diverged within the last
463 500 000 years[16]. We also found support for epistatic interactions between genes
464 located among the candidate barrier regions. Majority of genes in these regions were
465 predicted to form a single protein-protein interaction network, with interactions for
466 homologous proteins documented or predicted in other species. We were unlikely to
467 find this network by chance if compared to the genomic background of *D.*
468 *melanogaster*. However, any polymorphisms we were able to call within our pooled
469 sequencing data was likely to give significant enrichment of protein-protein
470 interactions using our analysis pipeline. Our annotation is based on *D. melanogaster*
471 homologs, which makes our pipeline biased towards conserved genes. If conserved
472 genes are also more likely to have evidence for protein-protein interactions, that could
473 lead to enrichment of interactions among both candidate barrier loci and random sets
474 of SNPs with our data and pipeline. However, there is no simple relationship between
475 sequence conservation and the number of protein-protein interactions[31], with evidence
476 for both positive[32] and negative[33] correlation between sequence conservation and
477 network connectivity. Our results highlight potential biases when extrapolating data
478 from model organisms into non-model systems. However, they also emphasize the
479 potential for protein-protein interactions, and so the possibility of epistatic
480 interactions, genome-wide in *Formica* ants. Our results are consistent with the idea of
481 multiple genes underlying hybrid breakdown that interact in a network instead of
482 multiple two-locus interactions. Functional and population studies are needed to test if

483 mismatching combinations of parental alleles at network loci lead to inviability, as
484 predicted under a DMI model[5,6].
485
486 Relatively few studies have investigated the effect of incompatibilities in networks or
487 incompatibility loci with more than one interaction (in comparison to two-locus
488 DMIs) for speciation, but see[7,34–36]. This is in stark contrast with our understanding of
489 molecular biology: genes and their products interact with several other genes, are part
490 of gene interaction networks, and are regulated by transcription factors. If
491 incompatibility loci interact in a network or pathway, it can have implications for the
492 evolution and stability of barriers to gene flow. For example, Porter and Johnson[37]
493 have shown that in contrast to two-locus DMI models, developmental pathways have
494 lower probabilities of divergence under high rates of gene flow. Yet, when sufficient
495 divergence has accumulated gene flow is less likely to reduce divergence if more loci
496 are involved in the pathway. Lindtke & Buerkle[38] have also modeled epistatic
497 interactions evolving in pathways, with results paralleling those of Porter & Johnson.
498 In the model by Lindtke & Buerkle pathways resulted in poor genome-wide barriers
499 to gene flow, but they could effectively prevent introgression near the genomic
500 locations of epistatically interacting loci. Moreover, DMIs with more than two
501 interacting loci were stronger barriers for gene flow than those with two interacting
502 loci[38]. The role of networks and pathways in the evolution of incompatibilities and
503 other "speciation genes" needs to be considered in future empirical and theoretical
504 studies of speciation.
505
506 Annotation of candidate barrier regions suggests genes in these regions play a role in
507 development, with 133 genes out of 268 investigated having the GO term
508 "developmental process". These results are consistent with our previous findings
509 where we show hybrid male breakdown during development in the *F. polyctena*–like
510 lineage[18]. Furthermore, developmental problems are a common cause of hybrid
511 breakdown[39–41]. Still, our analysis pipeline is likely to produce bias towards conserved
512 genes because it is based on *D. melanogaster* homologs, and developmental genes are
513 more likely to be conserved at the sequence level[42]. GO term "developmental
514 process" is not significantly enriched in the candidate barrier regions when compared
515 to random sets of SNPs in our data. Thus it is likely that additional gene functions
516 could be found if we were able to annotate the function of all open reading frames

517 within candidate barrier regions. Interestingly, our candidate barrier loci included
518 homologs of two major barrier loci in *Heliconius*: a member of the *Wnt* gene family
519 and *optix,* which are known to act as barriers to gene flow and are responsible for
520 variation in wing patterning between *Heliconius* races[8,43,44].
521
522 But why would a developmental gene network have diverged between the two
523 *Formica* species parental to the hybrids? Divergence could have been driven by drift
524 or differential adaptation between the parental species. The parental species have
525 likely diverged in allopatry and may now be adapted into different climatic
526 conditions, *F. aquilonia* being distributed in Northern Eurasia and higher altitudes of
527 Central Europe, while *F. polyctena* is likely adapted to warmer climate and occurs in
528 Central Europe and Southern parts of Fennoscandia and Russia[45]. While the species
529 have adapted into different climatic regimes it is plausible that their developmental
530 programs have adapted and diverged too. However, developmental programs can
531 diverge also at least partly by drift. Developmental systems drift, where homologous
532 characters develop via different regulation of genes or involve completely different
533 sets of genes is common[46,47].
534
535 *Signs of selection at candidate barrier loci and barrier leakage*
536 Previously we have shown antagonistic selection on ant hybrids, where introgressed
537 alleles were favored in female heterozygotes, but selected against in haploid males,
538 with strongest evidence of this in the *F. polyctena*–like lineage[17,18]. Our current
539 dataset shows a trend of increasing heterozygosity excess at candidate barrier loci
540 during female development, but this is not statistically significant after removal of
541 two outlier individuals likely to be early generation hybrids. However, we find a
542 significant excess of heterozygosity at the candidate barrier loci compared to the
543 random loci. This excess heterozygosity can be explained by two processes or their
544 combination. First, strong allele frequency differences between mothers and fathers
545 would result in high heterozygosity in diploid females without the action of selection
546 in the sampled generation. However, in this scenario initial differentiation between
547 sexes at candidate barrier loci was likely caused by selection (see below). Second,
548 selection that favors heterozygotes may have already acted on the candidate loci
549 before the larval stage we sampled. Our earlier study documented selection during
550 development using eggs and few days old larvae and compared them to adults,

551 whereas here we use 1-2 week old larvae and adults. Thus, in the current study we are
552 likely to miss any signal of selection acting early in development.
553
554 We assumed the candidate barrier loci to represent recessive incompatibilities and loci
555 linked to them, thus expecting to find evidence of negative selection in the haploid
556 males[17,18]. However, we find the opposite with introgressed alleles increasing in
557 frequency at the candidate barrier loci in the *F. aquilonia*–like males during
558 development. This is consistent with natural selection favoring introgression in these
559 males. The magnitude of change is comparable to that observed within a generation in
560 other systems, e.g. in stick insects for the color pattern locus[48]. Natural selection for,
561 instead of against introgression can be explained by sign epistasis, where the direction
562 of selection acting on a given allele depends on alleles at other loci within the
563 genome. Experimental evidence for sign epistasis is found in many systems, e.g.[49–51].
564
565 Selection for introgression within a generation in males may be connected to patterns
566 we observed between years: A proportion of the barrier loci showed barrier leakage
567 resulting in increase of introgressed alleles in males sampled 10 years apart. These
568 rising frequencies of introgressed alleles in *F. aquilonia*–like haploid males between
569 years can be explained by at least three hypotheses, which are not mutually exclusive.
570 First, the barriers to gene flow can be gradually breaking down in *F. aquilonia*–like
571 males, which could explain both within- and between-year patterns. Barrier breakage
572 could be due to recombination and subsequent formation of compatible combinations
573 of barrier loci in males. However, formation of compatible combinations may be less
574 likely if the candidate barrier loci are located in multiple chromosomes and interact in
575 a network[37,38], as our current results suggest. Barriers for gene flow could also break
576 down due to relaxed selection. Under this model the haplodiploid systems (and X-
577 chromosomes) can show fluctuating allele frequencies at candidate barrier loci,
578 comparable to those observed in our data, if sexes had different allele frequencies to
579 begin with. However, this hypothesis can only explain change between years and not
580 selection for introgressed alleles within a generation in males. Also, allele frequency
581 differences between sexes are not expected to be maintained without selection over
582 multiple generations. Second, a proportion of marker loci may have been hitchhiking
583 due to close genomic location with barrier loci and so missing from males in 2004,
584 but these hitchhikers are now becoming dissociated from the barrier loci via

recombination and consequently found in males in 2014. Again, this hypothesis cannot explain the significant increase of introgressed alleles within a generation in males. A third possibility is that the incompatibilities could be environment-dependent, which could explain both within-generation and between-years effect. Environment-dependence means that the negative effects of the incompatibilities would be expressed only in certain environments. This situation is comparable to genotype × genotype × environment interaction, where fitness effects of a genetic variant are dependent on the genomic background it is found in, as well as the external environment. Previous empirical evidence for environment-dependent incompatibilities comes e.g. from yeast[52] and *Drosophila*[53]. New genome-wide data needs to be collected over multiple years and generations in order to rigorously test the above hypotheses.

Barrier effects may be environment dependent

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[20,52,53]. 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 because it differs between the environments of the parental species and it 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 (Figure 7a & b), but due to the limited time-series this correlation cannot be tested statistically. 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.

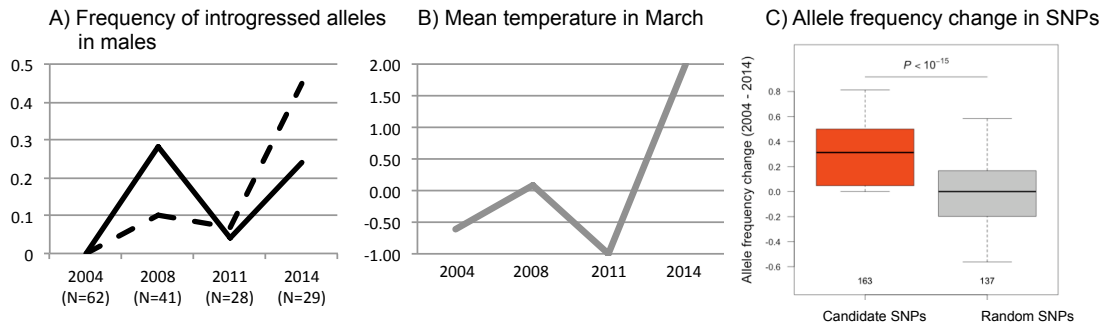

Figure 7. Frequency of introgressed alleles in adult *F. aquilonia*–like males appears to co-vary with yearly spring temperature. A) Frequency of alleles introgressed from *F. polyctena* to *F. aquilonia*–like males at two microsatellite loci (dashed FY13_190, straight FY15_222) in years 2004, 2008, 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.

Conclusions

Here we bridged the gap between genome scan studies and fitness by mapping candidate barrier loci between two recently diverged wood ant species and demonstrated signs of natural selection acting on candidate barrier regions. However, our results revealed barrier leakage that may depend on the environment. These results highlight the dynamic nature of the ant system that allows investigations into genomic and molecular consequences of hybridization, areas where many questions still remain to be answered.

Materials and Methods

Pooled genomic sequencing

We used pooled genomic sequencing to compare male and female genomes between *F. aquilonia*–like and *F. polyctena*–like hybrid lineages and to discover the candidate barrier loci (i.e. genomic regions putatively associated to hybrid male breakdown). We collected the samples used for pooled sequencing from the Långholmen hybrid population (Kulmuni et al. 2010) in the year 2004. The samples were freshly frozen and kept in -20°C and genomic DNA was extracted in the year 2010 from half a body using Qiagen kit. We sequenced altogether four pools of individuals, where each pool consisted of the following number of individuals; 1) 24 *F. aquilonia*–like males, 2) 25 *F. polyctena*–like males, 3) 24 *F. polyctena*–like females and 4) 24 *F. aquilonia*–like females. The sample concentrations were checked with Qubit and pooled into the four

647 pools in equal amounts. Each pool was sequenced with 100bp paired-end sequencing
648 on its own lane in Illumina HiSeq2000 in the Institute for Molecular Medicine
649 Finland (FIMM). This resulted in 46,106,000 to 108,204,481 total number of reads
650 per pool. We quality trimmed reads by removing up to 20 bp that had phred score <
651 20 using FASTX-Toolkit. Next, we made denovo assemblies of each of the four
652 samples with Soapdenovo trying out different kmer sizes (31, 41, 51, 61, 71) for each
653 assembly. The *F. polyctena*–like male assembly with kmer size of 41 was best in
654 terms of completeness and quality (genome size: 222.6 Mb, 327480 contigs, average
655 contig length: 679 bp, N50 = 1748 bp) and chosen as our reference assembly. We then
656 mapped each sample back to the *F. polyctena*–like male reference assembly after
657 removing contigs of the assembly shorter than 500bp using Bowtie2 v2.0.2. Reads
658 mapped in proper pairs and with a mapping quality superior to 20 were filtered using
659 samtools 1.4[54]. Filtered BAM files from the different pools were combined in a
660 mpileup file using samtools. Since coverage of the *F. aquilonia*–like male pool was
661 low (mean = 16, s.d. = 38), overlaps between read pairs were kept. For all SNPs (see
662 below) and in each pool, read counts were compared with or without filtering of read
663 pair overlaps using χ^2 tests as implemented in the poolseq R package[55]. Over all
664 pools, four SNPs displayed significant allele frequency change ($P < 0.05$, for
665 stringency no Bonferroni correction was applied) and were removed from the dataset
666 (Supplementary Figure 5). The mpileup file was then converted in a synchronized file
667 using Popoolation2[56]. Indels and their 5-bp flanking sequences were masked, and only
668 bi-allelic sites displaying a minimum base quality of 20, coverage between 20 and 60
669 for each population and a minor allele count of four across all populations were
670 considered. These steps led to the identification of 166,167 SNPs for which F_{ST}
671 estimates were computed from read count data using Popoolation2, adjusting for
672 differences in ploidy levels between haploid male and diploid female pools. These
673 estimates were compared with those obtained following a more recently developed
674 approach[57]: both methods provided similar results ($\rho = 0.96$) and identified a similar
675 number of SNPs differentially fixed in males (Popoolation2: 711; Poolfstat: 719,
676 including all the loci found using Popoolation2). Popoolation2 results were more
677 conservative (i.e, less differentially fixed SNPs) and were used for the rest of the
678 study. For the comparison between poolseq and SNP genotyping data sets, allele

679 counts were imputed from read counts using a maximum-likelihood approach[58],
680 considering the number of chromosomes sampled per pool.
681
682 *Gene Annotation, protein-protein interactions and GO term Enrichment Analysis*
683 We defined barrier loci as SNP positions where the male genomes (sampled in 2004)
684 from the *F. aquilonia*–like and *F. polyctena*–like lineages were fixed differently (i.e.
685 $F_{ST} = 1$, see Supplementary Figure 2), which led to identification of 711 SNPs. These
686 fall into to 610 assembled scaffolds. We created an automated pipeline to annotate
687 genomic regions around candidate barrier SNPs, taking advantage of the recent
688 release of the closely related *F. exsecta* genome[22]. First, using our assembly we
689 extracted a sequence of 5,000 bp centered on each of the 711 outlier SNPs. If the
690 contig was smaller than 5,000 bp, the full contig sequence was recovered. Since some
691 SNPs located on the same contig, and sometimes less than 5 kb apart, 639 unique
692 sequences were recovered and were blasted against the *F. exsecta* assembly. For each
693 queried sequence, hits covering less than 60% of its length were filtered out and the
694 best hit was kept per query based on e-value (over all queries, *e*-value $< 10^{-141}$). This
695 allowed to anchor 571 genomic regions on the *F. exsecta* assembly. The coordinates
696 of these regions were extended if needed to reach 5,000 bp, so that sampling effort
697 was equal among genomic regions. *F. exsecta* genes overlapping with best hits were
698 collected using the GenomeIntervals Bioconductor package v1.38[59]. Overall, 590
699 unique genes overlapped with our 571 genomic regions. Sixty-three of the candidate
700 genomic regions did not contain any annotated gene. The mean number of genes per
701 candidate genomic region was 1.4, and the maximum was six. For each gene, its *F.*
702 *exsecta* transcript sequence was recovered and blasted against *D. melanogaster* r6.15
703 proteins after filtering for the longest protein per gene. Alignments below 150 bp and
704 with less than 35% identity were filtered out and the best hit was kept for each query
705 based on e-value (over all queries, *e*-value $< 10^{-20}$). This pipeline recovered 317 *D.*
706 *melanogaster* genes whose homologs reside in candidate barrier regions. These
707 candidate barrier genes were used for subsequent analyses (e.g., GO and PPI
708 enrichment analyses). For a complete list of scaffolds annotated with their gene names
709 see Supplementary Table 1. GO term enrichment analysis was performed using the
710 Gene Ontology Consortium tool and protein interactions were analyzed using
711 STRING database and *D. melanogaster* homologs for our candidate genes. We
712 performed 1,000 simulations to assess whether the observed PPI enrichment indicated

713 by STRING was significantly different from what would be expected for a random set
714 of SNPs from our dataset. For each simulation, we randomly drew 711 SNPs from the
715 total dataset (166,167 SNPs) and annotated their flanking regions using the same
716 automated pipeline and parameter values as presented above. The number of genes
717 recovered per simulation varied between 267 and 368 (mean = 319). For all
718 simulations and empirical data, PPI enrichment values were computed with STRING
719 v10 using the STRINGdb Bioconductor package[24], setting the score threshold to 800.
720
721 We investigated the location of the candidate barrier SNPs blasting to the genome of
722 closely related *F. exsecta*[22] (277 Mb, 14617 scaffolds, N50 = 997654), as our own
723 genomic assembly is highly fragmented.
724
725 *Genotyping of candidate barrier and random SNPs*
726 To test for selection acting on candidate barrier SNPs we genotyped a set of candidate
727 and random SNPs from samples collected from the study population in 2014. These
728 samples were used to test if significant allele frequency changes occur between larval
729 and adult stages in candidate barrier SNPs but not in random SNPs, as expected if
730 these barrier SNPs mark genomic regions under selection. Genotyping was done at
731 individual level for a total of 196 individuals (Supplementary table 2). Samples were
732 randomly assigned to 96-well plates for genotyping.
733
734 Primer design and genotyping were done at LGC genomics using KASP genotyping
735 chemistry. We randomly chose 350 SNPs from the total SNP dataset. Out of these
736 180 were appropriate for primer design and genotyping. We further chose 183
737 candidate barrier SNPs out from the 711 SNPs identified previously. Candidate
738 barrier SNPs had F_{ST} of 1 between the male genomes but F_{ST} varied between females
739 at these loci. We aimed at variable F_{ST} estimates between females at the genotyped
740 barrier SNPs and initially chose the 50 SNPs with lowest F_{ST}, 50 SNPs with highest
741 F_{ST} and 100 randomly chosen from the remaining (Figure S1). Of these, 144 SNPs
742 were suitable for genotyping. Therefore, another 106 candidate barrier SNPs were
743 extracted, of which 76 passed the LGC primer design pipeline. In total 220 candidate
744 barrier SNPs were suitable for genotyping and from these we chose 183 SNPs
745 (Supplementary Figure 1). After removal of SNPs or individuals with more than 10%
746 missing data, diploid males from the *F. polyctena*–like lineage and three ambiguous

747 individuals (see Supplementary Figure 3), we were left with 181 individuals (for the
748 *F. polyctena*–like females, early stage: 27, late stage: 32, for the *F. aquilonia*–like
749 individuals, males early stage: 32, late stage: 32, females early stage: 31, late stage:
750 31) genotyped at 300 SNPs (137 random and 163 putative barrier loci).
751
752 *Analyses of allele and genotype frequencies*
753 F_{IS} values were estimated per genotyped locus and per group using the HierFstat
754 package[60]. We tested for significant differences in allele counts in *F. aquilonia*–like
755 males between larval and adult stages at candidate barrier SNPs compared to the
756 random SNPs using generalized linear mixed effects model in R[61]. We used locus
757 type (candidate or barrier) and developmental stage (larva or adult) as explanatory
758 variables and locus_ID as a random factor. To test if F_{IS} estimates were significantly
759 different at candidate barrier loci in females between larval and adult stages we used
760 linear mixed effects model[61] and locus type, developmental stage and group (*F.*
761 *polyctena* –like or *F. aquilonia*–like) as explanatory variables and locus_ID as a
762 random factor.
763
764 **Acknowledgements**
765 We thank Pekka Pamilo and Simon H. Martin for their valuable comments on the
766 manuscript and Petri Kemppainen for discussions. JK was supported by Human
767 Frontier Science Program Long-term fellowship and Academy of Finland grant nro.
768 309580.
769
770 **Author contributions**
771 JK and RB designed the study. JK, PN, LP, IS and KD analyzed the data. JK, PN, LP,
772 IS and RB wrote the manuscript.
773
774 **Data availability**
775 Data will be deposited into Dryad upon acceptance.
776
777 **References**
778 1. Mayr, E. *Populations, species, and evolution; an abridgment of Animal species*
779 *and evolution.* Belknap Press of Harvard University Press (1970).
780 2. Turner, T. L., Hahn, M. W. & Nuzhdin, S. V. Genomic Islands of Speciation in

781 Anopheles gambiae. *PLoS Biol.* **3,** e285 (2005).
782 3. Nosil, P. & Feder, J. L. Genomic divergence during speciation: Causes and
783 consequences. *Philos. Trans. R. Soc. B Biol. Sci.* **367,** 332–342 (2012).
784 4. Ravinet, M. *et al.* Interpreting the genomic landscape of speciation: a road map
785 for finding barriers to gene flow. *J. Evol. Biol.* **30,** 1450–1477 (2017).
786 5. Dobzhansky, T. Studies on Hybrid Sterility. II. Localization of Sterility Factors
787 in Drosophila Pseudoobscura Hybrids. *Genetics* **21,** 113–35 (1936).
788 6. Muller, H. J. Isolating Mechanisms, Evolution, and Temperature. *Biological*
789 *Symposia* 71–125 (1942).
790 7. Kulmuni, J. & Westram, A. M. Intrinsic incompatibilities evolving as a by-
791 product of divergent ecological selection: Considering them in empirical
792 studies on divergence with gene flow. *Mol. Ecol.* **26,** 3093–3103 (2017).
793 8. Martin, S. H. *et al.* Genome-wide evidence for speciation with gene flow in
794 Heliconius butterflies. *Genome Res.* 1817–1828 (2013).
795 9. Soria-Carrasco, V. *et al.* Stick insect genomes reveal natural selection's role in
796 parallel speciation. *Science* **344,** 738–42 (2014).
797 10. Burri, R. *et al.* Linked selection and recombination rate variation drive the
798 evolution of the genomic landscape of differentiation across the speciation
799 continuum of Ficedula flycatchers. *Genome Res.* **25,** 1656–65 (2015).
800 11. Twyford, A. D. & Friedman, J. Adaptive divergence in the monkey flower
801 *Mimulus guttatus* is maintained by a chromosomal inversion. *Evolution.* **69,**
802 1476–1486 (2015).
803 12. Charlesworth, B. Background selection and patterns of genetic diversity in
804 Drosophila melanogaster. *Genet. Res.* **68,** 131–149 (1996).
805 13. Cruickshank, T. E. & Hahn, M. W. Reanalysis suggests that genomic islands of
806 speciation are due to reduced diversity, not reduced gene flow. *Mol. Ecol.* **23,**
807 3133–3157 (2014).
808 14. Wolf, J. B. W. & Ellegren, H. Making sense of genomic islands of
809 differentiation in light of speciation. *Nat. Rev. Genet.* **18,** 87–100 (2017).
810 15. Hofer, T., Foll, M. & Excoffier, L. Evolutionary forces shaping genomic
811 islands of population differentiation in humans. *BMC Genomics* **13,** 107
812 (2012).
813 16. Goropashnaya, A. V., Fedorov, V. B., Seifert, B. & Pamilo, P. Phylogenetic
814 relationships of Palaearctic Formica species (hymenoptera, Formicidae) based

815 on mitochondrial cytochrome b sequences. *PLoS One* **7,** (2012).
816 17. Kulmuni, J., Seifert, B. & Pamilo, P. Segregation distortion causes large-scale
817 differences between male and female genomes in hybrid ants. *Proc. Natl. Acad.*
818 *Sci. U. S. A.* **107,** 7371–7376 (2010).
819 18. Kulmuni, J. & Pamilo, P. Introgression in hybrid ants is favored in females but
820 selected against in males. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 1323045111-
821 (2014).
822 19. Ghenu, A.-H., Blanckaert, A., Butlin, R. K., Kulmuni, J. & Bank, C. Conflict
823 between heterozygote advantage and hybrid incompatibility in haplodiploids
824 (and sex chromosomes). *Mol. Ecol.* **19**, 3935-3949 (2018).
825 20. Schumer, M. *et al.* Assortative mating and persistent reproductive isolation in
826 hybrids. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 10936-10941 (2017).
827 21. Beresford, J. *et al.* Widespread hybridization within mound-building wood ants
828 in Southern Finland results in cytonuclear mismatches and potential for sex-
829 specific hybrid breakdown. *Mol. Ecol.* **26,** 4013–4026 (2017).
830 22. Dhaygude, K., Nair, A., Johansson, H., Wurm, Y. & Sundström, L. The first
831 draft genomes of the ant Formica exsecta, and its Wolbachia endosymbiont
832 reveal extensive gene transfer from endosymbiont to host. *bioRxiv* 436691
833 (2018).
834 23. Rosengren, M., Rosengren, R. & Söderlund, V. Chromosome numbers in the
835 genus Formica with special reference to the taxonomical position of Formica
836 uralensis Ruzsk. and Formica truncorum Fabr. *Hereditas* **92,** 321–325 (1980).
837 24. Szklarczyk, D. *et al.* STRING v10: protein–protein interaction networks,
838 integrated over the tree of life. *Nucleic Acids Res.* **43,** D447–D452 (2015).
839 25. Rellstab, C., Zoller, S., Tedder, A., Gugerli, F. & Fischer, M. C. Validation of
840 SNP Allele Frequencies Determined by Pooled Next-Generation Sequencing in
841 Natural Populations of a Non-Model Plant Species. *PLoS One* **8,** e80422
842 (2013).
843 26. Fracassetti, M., Griffin, P. C. & Willi, Y. Validation of Pooled Whole-Genome
844 Re-Sequencing in Arabidopsis lyrata. *PLoS One* **10,** e0140462 (2015).
845 27. Lexer, C. *et al.* Genomic admixture analysis in European Populus spp. reveals
846 unexpected patterns of reproductive isolation and mating. *Genetics* **186,** 699–
847 712 (2010).
848 28. McGaugh, S. E. & Noor, M. A. F. Genomic impacts of chromosomal

849 inversions in parapatric Drosophila species. *Philos. Trans. R. Soc. B Biol. Sci.*
850 **367,** 422–429 (2012).
851 29. Jones, F. C. *et al.* The genomic basis of adaptive evolution in threespine
852 sticklebacks. *Nature* **484,** 55–61 (2012).
853 30. Byers, K. J. R. P., Xu, S. & Schlüter, P. M. Molecular mechanisms of
854 adaptation and speciation: why do we need an integrative approach? *Mol. Ecol.*
855 **26,** 277–290 (2017).
856 31. Jordan, I. K., Wolf, Y. I. & Koonin, E. V. No simple dependence between
857 protein evolution rate and the number of protein-protein interactions: only the
858 most prolific interactors tend to evolve slowly. *BMC Evol. Biol.* **3,** 1 (2003).
859 32. Luisi, P. *et al.* Recent positive selection has acted on genes encoding proteins
860 with more interactions within the whole human interactome. *Genome Biol.*
861 *Evol.* **7,** 1141–1154 (2015).
862 33. Josephs, E. B., Wright, S. I., Stinchcombe, J. R. & Schoen, D. J. The
863 Relationship between Selection, Network Connectivity, and Regulatory
864 Variation within a Population of Capsella grandiflora. *Genome Biol. Evol.* **9,**
865 1099–1109 (2017).
866 34. Bomblies, K. Doomed lovers: mechanisms of isolation and incompatibility in
867 plants. *Annu. Rev. Plant Biol.* **61,** 109–124 (2010).
868 35. Chae, E. *et al.* Species-wide genetic incompatibility analysis identifies immune
869 genes as hot spots of deleterious epistasis. *Cell* **159,** 1341–1351 (2014).
870 36. Turner, L. M., White, M. a., Tautz, D. & Payseur, B. a. Genomic Networks of
871 Hybrid Sterility. *PLoS Genet.* **10,** 18–22 (2014).
872 37. Porter, A. H. & Johnson, N. A. Speciation despite Gene Flow When
873 Developmental Pathways Evolve *Evolution* **56,** 2103–2111 (2002).
874 38. Lindtke, D. & Buerkle, C. A. The genetic architecture of hybrid
875 incompatibilities and their effect on barriers to introgression in secondary
876 contact. *Evolution* **69**, 1987–2004 (2015).
877 39. Coyne, J. A. & Orr, H. A. *Speciation*. (Sinauer Associates, 2004).
878 40. Rogers, S. M. & Bernatchez, L. The genetic basis of intrinsic and extrinsic
879 post-zygotic reproductive isolation jointly promoting speciation in the lake
880 whitefish species complex (Coregonus clupeaformis). *J. Evol. Biol.* **19,** 1979–
881 1994 (2006).
882 41. McDaniel, S. F., Willis, J. H. & Shaw, A. J. The genetic basis of

883 developmental abnormalities in interpopulation hybrids of the moss Ceratodon
884 purpureus. *Genetics* **179,** 1425–35 (2008).
885 42. Nei, M. The new mutation theory of phenotypic evolution. *Proc. Natl. Acad.*
886 *Sci. U. S. A.* **104,** 12235–42 (2007).
887 43. Mazo-Vargas, A. *et al.* Macroevolutionary shifts ofWntAfunction potentiate
888 butterfly wing-pattern diversity. *Proc. Natl. Acad. Sci. U. S. A.* **114,** 10701–
889 10706 (2017).
890 44. Reed, R. D. *et al.* optix Drives the Repeated Convergent Evolution of Butterfly
891 Wing Pattern Mimicry. *Science* **333,** 1137–1141 (2011).
892 45. Stockan, J. A., Robinson, E. J. H., Trager, J. C., Yao, I. & Seifert, B. in *Wood*
893 *Ant Ecology and Conservation* (eds. Stockan, J. A. & Robinson, E. J. H.) 1–36
894 (Cambridge University Press, 2016).
895 46. True, J. R. & Haag, E. S. Developmental system drift and flexibility in
896 evolutionary trajectories. *Evol. Dev.* **3,** 109–19
897 47. Pavlicev, M. & Wagner, G. P. A model of developmental evolution: Selection,
898 pleiotropy and compensation. *Trends Ecol. Evol.* **27,** 316–322 (2012).
899 48. Nosil, P. *et al.* Natural selection and the predictability of evolution in Timema
900 stick insects. **770,** 765–770 (2018).
901 49. de Visser, J. a. G. M., Cooper, T. F. & Elena, S. F. The causes of epistasis.
902 *Proc. R. Soc. B Biol. Sci.* **278,** 3617–3624 (2011).
903 50. Zee, P. C. *et al.* A Shift from magnitude to sign epistasis during adaptive
904 evolution of a bacterial social trait. *Evolution* **68,** 2701–2708 (2014).
905 51. Ono, J., Gerstein, A. C. & Otto, S. P. Widespread Genetic Incompatibilities
906 between First-Step Mutations during Parallel Adaptation of Saccharomyces
907 cerevisiae to a Common Environment. PLOS Biology 1–26 (2017).
908 52. Hou, J., Friedrich, A., Gounot, J.-S. & Schacherer, J. Comprehensive survey of
909 condition-specific reproductive isolation reveals genetic incompatibility in
910 yeast. *Nat. Commun.* **6,** 7214 (2015).
911 53. Hoekstra, L. A., Siddiq, M. A. & Montooth, K. L. Pleiotropic effects of a
912 mitochondrial-nuclear incompatibility depend upon the accelerating effect of
913 temperature in Drosophila. *Genetics* **195,** 1129–39 (2013).
914 54. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools.
915 *Bioinformatics* **25,** 2078–2079 (2009).
916 55. Taus, T., Futschik, A. & Schlötterer, C. Quantifying Selection with Pool-Seq

Time Series Data. *Mol. Biol. Evol.* **34,** 3023–3034 (2017).

56. Kofler, R., Pandey, R. V. & Schlötterer, C. PoPoolation2: Identifying differentiation between populations using sequencing of pooled DNA samples (Pool-Seq). *Bioinformatics* **27,** 3435–3436 (2011).

57. Hivert, V., Leblois, R., Petit, E. J., Gautier, M. & Vitalis, R. Measuring Genetic Differentiation from Pool-seq Data. *Genetics* **210,** 315–330 (2018).

58. Leblois, R. *et al.* Deciphering the demographic history of allochronic differentiation in the pine processionary moth *Thaumetopoea pityocampa*. *Mol. Ecol.* **27,** 264–278 (2018).

59. Gagneur, J., Toedling, J., Bourgon, R. & Delhomme, N. genomeIntervals: Operations on genomic intervals. R package version 1.38.0. (2018).

60. GOUDET, J. hierfstat, a package for r to compute and test hierarchical F-statistics. *Mol. Ecol. Notes* **5,** 184–186 (2005).

61. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models Using **lme4**. *J. Stat. Softw.* **67,** 1–48 (2015).