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

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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

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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. 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.

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. 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. 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; 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. polycrana* (previously referred to as R group). 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. polycrana*-like lineage, where over 90% of male eggs were estimated to die. 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. 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](image_url)

**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. = $4.66 \times 10^{-4}$) between the males, and 0.07 (s.e. = $2.27 \times 10^{-4}$) 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. = $5.83 \times 10^{-3}$, 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
We used the genome of the closely-related *Formica exsecta* (277 Mb, 14,617 scaffolds, N50 = 997,654), which is better assembled compared to the poolseq genome assembly, to map the location of candidate barrier loci. *F. exsecta* is estimated to have diverged from the parental species of our hybrid population 5 Mya. We used the 610 contigs containing candidate barrier SNPs as queries and searched the *F. exsecta* genome assembly using BlastN. If our query scaffold was larger than 5 000 bp we used +/- 2 500 bp surrounding the candidate barrier SNP as query. Our candidate barrier loci map to a total of 134 *F. exsecta* contigs, with a cumulative coverage of 56.2 % of the *F. exsecta* genome, both of which are significantly less than for a similar number of random SNPs in our data set (Supplementary Figure 2). Haploid chromosome number varies between 26 and 28 in *Formica*. Assuming evenly-sized chromosomes our candidate barrier loci are likely to be situated in over 10 chromosomes.

**Candidate barrier loci form a network of interacting proteins**

Next, we were interested in the possible functions of our candidate barrier loci, as they can be informative about the putative selection pressures and mechanisms of hybrid male breakdown. We annotated each of the 610 contigs containing candidate barrier SNPs, first extracting the transcripts of the genes located in those regions from the closely related *F. exsecta*. Out of 571 candidate barrier regions (see Material and Methods; each region was defined as 5000 bp around the candidate barrier SNP) that we were able to anchor in *F. exsecta* (all blastn e-values < 10^{-141}), we recovered 590 unique genes. Sixty-three of the candidate genomic regions did not contain any annotated gene. The mean number of genes per candidate genomic region was 1.4, and the maximum was six. For each gene, its *F. exsecta* transcript sequence was recovered and blasted (blastx) against *Drosophila melanogaster* r6.15 proteins recovering 317 genes, whose homologs reside in candidate barrier regions (Supplementary Table 1).

The genetic model for hybrid breakdown suggests inviability and sterility are caused by dysfunction and incompatibilities between diverged genes from the parental species. We thus asked if there was any evidence for protein-protein interactions between the gene products in candidate barrier regions using the recovered *D. melanogaster* proteins and the STRING database, 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.
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” ($p = 3.5 \times 10^{-18}$), “system development” ($p = 3.5 \times 10^{-18}$), and “single-organism developmental process” ($p = 4.03 \times 10^{-18}$). Similarly, the top three molecular functions are “ion binding” ($p = 8.55 \times 10^{-6}$), “protein binding” ($p = 3.67 \times 10^{-5}$) and “calcium ion binding” ($p = 8.74 \times 10^{-5}$). 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 set of SNPs drawn from our data, as all of them are found in over 5% of our simulations.

*Testing for signs of selection at candidate barrier loci in a natural population*

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

The SNP genotyping confirmed that within the hybrid population, *F. polycrvena*–like and *F. aquilonia*–like lineages are genetically distinct from one another (Supplementary Figure 3). We found two small female larvae, which were genetically intermediate and had unique combinations of putative parental alleles from the two
lineages suggesting they were early-generation hybrids between the lineages
(Supplementary Figure 3). The *F. polyctena*-like lineage was previously shown to
experience stronger selection than the *F. aquilonia*-like lineage. Especially, over 90
% of the male eggs laid in the *F. polyctena*-like lineage were estimated to be inviable
due to incompatible combinations of introgressed and putative ancestral alleles at
microsatellite loci\(^{18}\). Unfortunately, we were able to sample only 8 males from this
lineage, five of which turned out to be diploid, thus *F. polyctena*-like males were
excluded from the analysis. The following analyses were performed for *F. polyctena-
like females, *F. aquilonia*-like males and *F. aquilonia*-like females.

First we asked if adult allele frequencies inferred from the pooled sequencing
correlated with adult allele frequencies estimated from the SNP genotyping data, as
the two data sets have been collected ten years apart and allele frequencies were
estimated in different ways. Over all the genotyped SNPs, allele frequencies between
the two data sets correlated well in *F. polyctena*-like females (Spearman’s
correlation, \(\rho = 0.87, \text{CI}_{95} = [0.82, 0.90], p < 0.001\)) and in *F. aquilonia*-like females
\((\rho = 0.91, \text{CI}_{95} = [0.88, 0.94], p < 0.001)\) (Figure 3). The correlation was lower for the
males with a Spearman’s correlation coefficient of 0.71 (\(\text{CI}_{95} = [0.64, 0.76], p <
0.001\)) between adult allele frequencies estimated from pooled sequencing and
genotyping (Figure 3). The lower correlation in males compared to females can be
explained, at least in part, by the lower number of sampled chromosomes for
(haploid) males compared to (diploid) females creating a larger error in the estimated
allele frequencies. Overall, correlations between allele frequency estimates are in line
with other studies focusing on the accuracy of allele frequency estimates derived from
poolseq\(^{25,26}\) and suggest pooled sequencing and SNP genotyping gave similar results.
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. polycrysta*-like to *F. aquilonia*-like, blue: allele introgressed from *F. aquilonia*-like to *F. polycrysta*-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. polycrysta*–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. polycrysta*-like to *F. aquilonia*-like, blue: allele introgressed from *F. aquilonia*-like to *F. polycrysta*-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*\(_{\text{IS}}\) estimates (Table 1). Indeed, there was a tendency for more negative mean *F*\(_{\text{IS}}\) 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                 |

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
selection, because too few *F. polycytena*–like males were sampled from the population. The low occurrence of these males is consistent with their estimated high mortality\(^1\). 

Significant variation in the strength of barriers through time

Introgressed (i.e., *F. polycytena*–like) alleles were absent from adult *F. aquilonia*–like males at 163 candidate barrier loci in year 2004 (allele frequency of zero in the poolseq data). However, in year 2014 significant proportion of these loci harbored alleles introgressed from the *F. polycytena*–like lineage (123 loci, i.e. 75.5%) to *F. aquilonia*–like males. The mean frequency of introgressed alleles in these males was 0.36, the most common introgressed allele being at a frequency of 0.81. We observed no population substructure within the *F. aquilonia*–like lineage (Supplementary Figures 3 and 4). Accordingly, sampling different sub-populations within a lineage at different years is unlikely to drive increase of introgressed allele frequencies between 2004 and 2014 in our samples of *F. aquilonia*–like males. Increase of introgressed alleles in males is not likely to be caused by errors in male poolseq-based allele frequency estimates either. All but one of the candidate barrier SNPs (33 out of 34 SNPs) that were initially fixed in both adult males and females of the *F. aquilonia*–like lineage in the pooled sequencing data (year 2004) were still fixed in the genotyping data (year 2014), showing consistency across data sets. If differences between allele frequency estimates from 2004 and 2014 were caused by errors in male poolseq allele frequency estimates, this should affect all SNP loci equally. However, this is not the case, as only candidate barrier SNPs that were polymorphic in females of the *F. aquilonia*–like lineage have now “leaked” into *F. aquilonia*–like males. Our results thus suggest that in year 2014 a proportion of the barrier loci are “leaking” in *F. aquilonia*–like males. At these loci introgressed alleles were on average selected for in males as evidenced by significant frequency increase between larval and adult stages (see above), a pattern opposite to previous observations\(^{17,18}\).

Using microsatellite genotyping, we further verified that increasing frequencies of introgressed alleles at candidate barrier SNPs in *F. aquilonia*–like males did not represent technical artifacts. Specifically, we genotyped the individuals collected in 2014 and used in SNP genotyping with nine previously used microsatellite markers\(^7,18\) and compared them to samples collected in 2004, 2008 and 2011 (Supplementary 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. polycytena*-like lineage that were missing from them in 2004 (Table 2). Specifically, the frequencies of two microsatellite alleles (FY3 and FY15) introgressed from *F. polycytena* to *F. aquilonia*-like males have fluctuated over the years (Table 2). Similar leakage of alleles introgressed from *F. aquilonia* to the *F. polycytena*—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 ref28.

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.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 approach4,30. 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.
Surprisingly, selection acts in a direction opposite to our expectation, favoring introgression in males. Thus, the ant system is dynamic and not likely to be in an equilibrium state, as at least a proportion of the barriers showed leakage in 2014 compared to 2004.

*Considering barrier loci that evolve in a network*

Hybrid breakdown is expected to result from deleterious epistatic interactions (i.e. intrinsic incompatibilities) between diverged genes of the parental species. However, two fundamental questions about incompatibilities remain to be answered. First, is hybrid breakdown based on few or many loci? Second, if breakdown is based on many loci do these represent multiple two-locus interactions or do incompatibility loci interact in networks or pathways? Our results provide insights into both of these questions. We found that candidate barrier and hitchhiking loci likely cover a large proportion of the genome in two *Formica* ant species, which diverged within the last 500,000 years. We also found support for epistatic interactions between genes located among the candidate barrier regions. Majority of genes in these regions were predicted to form a single protein-protein interaction network, with interactions for homologous proteins documented or predicted in other species. We were unlikely to find this network by chance if compared to the genomic background of *D. melanogaster*. However, any polymorphisms we were able to call within our pooled sequencing data was likely to give significant enrichment of protein-protein interactions using our analysis pipeline. Our annotation is based on *D. melanogaster* homologs, which makes our pipeline biased towards conserved genes. If conserved genes are also more likely to have evidence for protein-protein interactions, that could lead to enrichment of interactions among both candidate barrier loci and random sets of SNPs with our data and pipeline. However, there is no simple relationship between sequence conservation and the number of protein-protein interactions, with evidence for both positive and negative correlation between sequence conservation and network connectivity. Our results highlight potential biases when extrapolating data from model organisms into non-model systems. However, they also emphasize the potential for protein-protein interactions, and so the possibility of epistatic interactions, genome-wide in *Formica* ants. Our results are consistent with the idea of multiple genes underlying hybrid breakdown that interact in a network instead of multiple two-locus interactions. Functional and population studies are needed to test if
mismatching combinations of parental alleles at network loci lead to inviability, as predicted under a DMI model\textsuperscript{5,6}.

Relatively few studies have investigated the effect of incompatibilities in networks or incompatibility loci with more than one interaction (in comparison to two-locus DMIs) for speciation, but see\textsuperscript{7,34–36}. This is in stark contrast with our understanding of molecular biology: genes and their products interact with several other genes, are part of gene interaction networks, and are regulated by transcription factors. If incompatibility loci interact in a network or pathway, it can have implications for the evolution and stability of barriers to gene flow. For example, Porter and Johnson\textsuperscript{37} have shown that in contrast to two-locus DMI models, developmental pathways have lower probabilities of divergence under high rates of gene flow. Yet, when sufficient divergence has accumulated gene flow is less likely to reduce divergence if more loci are involved in the pathway. Lindtke & Buerkle\textsuperscript{38} have also modeled epistatic interactions evolving in pathways, with results paralleling those of Porter & Johnson. In the model by Lindtke & Buerkle pathways resulted in poor genome-wide barriers to gene flow, but they could effectively prevent introgression near the genomic locations of epistatically interacting loci. Moreover, DMIs with more than two interacting loci were stronger barriers for gene flow than those with two interacting loci\textsuperscript{38}. The role of networks and pathways in the evolution of incompatibilities and other “speciation genes” needs to be considered in future empirical and theoretical studies of speciation.

Annotation of candidate barrier regions suggests genes in these regions play a role in development, with 133 genes out of 268 investigated having the GO term “developmental process”. These results are consistent with our previous findings where we show hybrid male breakdown during development in the F. polycnetna–like lineage\textsuperscript{18}. Furthermore, developmental problems are a common cause of hybrid breakdown\textsuperscript{39–41}. Still, our analysis pipeline is likely to produce bias towards conserved genes because it is based on D. melanogaster homologs, and developmental genes are more likely to be conserved at the sequence level\textsuperscript{42}. GO term “developmental process” is not significantly enriched in the candidate barrier regions when compared to random sets of SNPs in our data. Thus it is likely that additional gene functions could be found if we were able to annotate the function of all open reading frames.
within candidate barrier regions. Interestingly, our candidate barrier loci included homologs of two major barrier loci in *Heliconius*: a member of the *Wnt* gene family and *optix*, which are known to act as barriers to gene flow and are responsible for variation in wing patterning between *Heliconius* races. But why would a developmental gene network have diverged between the two *Formica* species parental to the hybrids? Divergence could have been driven by drift or differential adaptation between the parental species. The parental species have likely diverged in allopatry and may now be adapted into different climatic conditions, *F. aquilonia* being distributed in Northern Eurasia and higher altitudes of Central Europe, while *F. polyctena* is likely adapted to warmer climate and occurs in Central Europe and Southern parts of Fennoscandia and Russia. While the species have adapted into different climatic regimes it is plausible that their developmental programs have adapted and diverged too. However, developmental programs can diverge also at least partly by drift. Developmental systems drift, where homologous characters develop via different regulation of genes or involve completely different sets of genes is common.

**Signs of selection at candidate barrier loci and barrier leakage**

Previously we have shown antagonistic selection on ant hybrids, where introgressed alleles were favored in female heterozygotes, but selected against in haploid males, with strongest evidence of this in the *F. polyctena*-like lineage. Our current dataset shows a trend of increasing heterozygosity excess at candidate barrier loci during female development, but this is not statistically significant after removal of two outlier individuals likely to be early generation hybrids. However, we find a significant excess of heterozygosity at the candidate barrier loci compared to the random loci. This excess heterozygosity can be explained by two processes or their combination. First, strong allele frequency differences between mothers and fathers would result in high heterozygosity in diploid females without the action of selection in the sampled generation. However, in this scenario initial differentiation between sexes at candidate barrier loci was likely caused by selection (see below). Second, selection that favors heterozygotes may have already acted on the candidate loci before the larval stage we sampled. Our earlier study documented selection during development using eggs and few days old larvae and compared them to adults,
whereas here we use 1-2 week old larvae and adults. Thus, in the current study we are likely to miss any signal of selection acting early in development.

We assumed the candidate barrier loci to represent recessive incompatibilities and loci linked to them, thus expecting to find evidence of negative selection in the haploid males\textsuperscript{17,18}. However, we find the opposite with introgressed alleles increasing in frequency at the candidate barrier loci in the \textit{F. aquilonia}–like males during development. This is consistent with natural selection favoring introgression in these males. The magnitude of change is comparable to that observed within a generation in other systems, e.g. in stick insects for the color pattern locus\textsuperscript{48}. Natural selection for, instead of against introgression can be explained by sign epistasis, where the direction of selection acting on a given allele depends on alleles at other loci within the genome. Experimental evidence for sign epistasis is found in many systems, e.g.\textsuperscript{49–51}.

Selection for introgression within a generation in males may be connected to patterns we observed between years: A proportion of the barrier loci showed barrier leakage resulting in increase of introgressed alleles in males sampled 10 years apart. These rising frequencies of introgressed alleles in \textit{F. aquilonia}–like haploid males between years can be explained by at least three hypotheses, which are not mutually exclusive. First, the barriers to gene flow can be gradually breaking down in \textit{F. aquilonia}–like males, which could explain both within- and between-year patterns. Barrier breakage could be due to recombination and subsequent formation of compatible combinations of barrier loci in males. However, formation of compatible combinations may be less likely if the candidate barrier loci are located in multiple chromosomes and interact in a network\textsuperscript{37,38}, as our current results suggest. Barriers for gene flow could also break down due to relaxed selection. Under this model the haplodiploid systems (and X-chromosomes) can show fluctuating allele frequencies at candidate barrier loci, comparable to those observed in our data, if sexes had different allele frequencies to begin with. However, this hypothesis can only explain change between years and not selection for introgressed alleles within a generation in males. Also, allele frequency differences between sexes are not expected to be maintained without selection over multiple generations. Second, a proportion of marker loci may have been hitchhiking due to close genomic location with barrier loci and so missing from males in 2004, 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 and Drosophila. 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. 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
pools in equal amounts. Each pool was sequenced with 100bp paired-end sequencing on its own lane in Illumina HiSeq2000 in the Institute for Molecular Medicine Finland (FIMM). This resulted in 46,106,000 to 108,204,481 total number of reads per pool. We quality trimmed reads by removing up to 20 bp that had phred score < 20 using FASTX-Toolkit. Next, we made denovo assemblies of each of the four samples with Soapdenovo trying out different kmer sizes (31, 41, 51, 61, 71) for each assembly. The *F. polyctena*–like male assembly with kmer size of 41 was best in terms of completeness and quality (genome size: 222.6 Mb, 327480 contigs, average contig length: 679 bp, N50 = 1748 bp) and chosen as our reference assembly. We then mapped each sample back to the *F. polyctena*–like male reference assembly after removing contigs of the assembly shorter than 500bp using Bowtie2 v2.0.2. Reads mapped in proper pairs and with a mapping quality superior to 20 were filtered using samtools 1.4. Filtered BAM files from the different pools were combined in a mpileup file using samtools. Since coverage of the *F. aquilonia*–like male pool was low (mean = 16, s.d. = 38), overlaps between read pairs were kept. For all SNPs (see below) and in each pool, read counts were compared with or without filtering of read pair overlaps using \( \chi^2 \) tests as implemented in the poolseq R package. Over all pools, four SNPs displayed significant allele frequency change (\( P < 0.05 \), for stringency no Bonferroni correction was applied) and were removed from the dataset (Supplementary Figure 5). The mpileup file was then converted in a synchronized file using Popoolation2. Indels and their 5-bp flanking sequences were masked, and only bi-allelic sites displaying a minimum base quality of 20, coverage between 20 and 60 for each population and a minor allele count of four across all populations were considered. These steps led to the identification of 166,167 SNPs for which \( F_{ST} \) estimates were computed from read count data using Popoolation2, adjusting for differences in ploidy levels between haploid male and diploid female pools. These estimates were compared with those obtained following a more recently developed approach: both methods provided similar results (\( \rho = 0.96 \)) and identified a similar number of SNPs differentially fixed in males (Popoolation2: 711; Poolfstat: 719, including all the loci found using Popoolation2). Popoolation2 results were more conservative (i.e, less differentially fixed SNPs) and were used for the rest of the study. For the comparison between poolseq and SNP genotyping data sets, allele
counts were imputed from read counts using a maximum-likelihood approach\textsuperscript{58}, considering the number of chromosomes sampled per pool.

Gene Annotation, protein-protein interactions and GO term Enrichment Analysis

We defined barrier loci as SNP positions where the male genomes (sampled in 2004) from the F. aquilonia–like and F. polycenæ–like lineages were fixed differently (i.e. $F_{ST} = 1$, see Supplementary Figure 2), which led to identification of 711 SNPs. These fall into 610 assembled scaffolds. We created an automated pipeline to annotate genomic regions around candidate barrier SNPs, taking advantage of the recent release of the closely related F. exsecta genome\textsuperscript{22}. First, using our assembly we extracted a sequence of 5,000 bp centered on each of the 711 outlier SNPs. If the contig was smaller than 5,000 bp, the full contig sequence was recovered. Since some SNPs located on the same contig, and sometimes less than 5 kb apart, 639 unique sequences were recovered and were blasted against the F. exsecta assembly. For each queried sequence, hits covering less than 60% of its length were filtered out and the best hit was kept per query based on e-value (over all queries, $e$-value < 10\textsuperscript{-141}). This allowed to anchor 571 genomic regions on the F. exsecta assembly. The coordinates of these regions were extended if needed to reach 5,000 bp, so that sampling effort was equal among genomic regions. F. exsecta genes overlapping with best hits were collected using the GenomeIntervals Bioconductor package v1.38\textsuperscript{59}. Overall, 590 unique genes overlapped with our 571 genomic regions. Sixty-three of the candidate genomic regions did not contain any annotated gene. The mean number of genes per candidate genomic region was 1.4, and the maximum was six. For each gene, its F. exsecta transcript sequence was recovered and blasted against D. melanogaster r6.15 proteins after filtering for the longest protein per gene. Alignments below 150 bp and with less than 35% identity were filtered out and the best hit was kept for each query based on e-value (over all queries, $e$-value < 10\textsuperscript{-20}). This pipeline recovered 317 D. melanogaster genes whose homologs reside in candidate barrier regions. These candidate barrier genes were used for subsequent analyses (e.g., GO and PPI enrichment analyses). For a complete list of scaffolds annotated with their gene names see Supplementary Table 1. GO term enrichment analysis was performed using the Gene Ontology Consortium tool and protein interactions were analyzed using STRING database and D. melanogaster homologs for our candidate genes. We performed 1,000 simulations to assess whether the observed PPI enrichment indicated
by STRING was significantly different from what would be expected for a random set of SNPs from our dataset. For each simulation, we randomly drew 711 SNPs from the total dataset (166,167 SNPs) and annotated their flanking regions using the same automated pipeline and parameter values as presented above. The number of genes recovered per simulation varied between 267 and 368 (mean = 319). For all simulations and empirical data, PPI enrichment values were computed with STRING v10 using the STRINGdb Bioconductor package, setting the score threshold to 800.

We investigated the location of the candidate barrier SNPs blasting to the genome of closely related *F. exsecta* (277 Mb, 14617 scaffolds, N50 = 997654), as our own genomic assembly is highly fragmented.

*Genotyping of candidate barrier and random SNPs*

To test for selection acting on candidate barrier SNPs we genotyped a set of candidate and random SNPs from samples collected from the study population in 2014. These samples were used to test if significant allele frequency changes occur between larval and adult stages in candidate barrier SNPs but not in random SNPs, as expected if these barrier SNPs mark genomic regions under selection. Genotyping was done at individual level for a total of 196 individuals (Supplementary table 2). Samples were randomly assigned to 96-well plates for genotyping.

Primer design and genotyping were done at LGC genomics using KASP genotyping chemistry. We randomly chose 350 SNPs from the total SNP dataset. Out of these 180 were appropriate for primer design and genotyping. We further chose 183 candidate barrier SNPs out from the 711 SNPs identified previously. Candidate barrier SNPs had $F_{ST}$ of 1 between the male genomes but $F_{ST}$ varied between females at these loci. We aimed at variable $F_{ST}$ estimates between females at the genotyped barrier SNPs and initially chose the 50 SNPs with lowest $F_{ST}$, 50 SNPs with highest $F_{ST}$ and 100 randomly chosen from the remaining (Figure S1). Of these, 144 SNPs were suitable for genotyping. Therefore, another 106 candidate barrier SNPs were extracted, of which 76 passed the LGC primer design pipeline. In total 220 candidate barrier SNPs were suitable for genotyping and from these we chose 183 SNPs (Supplementary Figure 1). After removal of SNPs or individuals with more than 10% missing data, diploid males from the *F. polycotena*–like lineage and three ambiguous
individuals (see Supplementary Figure 3), we were left with 181 individuals (for the
*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).

Analyses of allele and genotype frequencies

*F*$_{IS}$ values were estimated per genotyped locus and per group using the HierFstat
package\textsuperscript{60}. 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\textsuperscript{61}. We used locus
type (candidate or barrier) and developmental stage (larva or adult) as explanatory
variables and locus\_ID as a random factor. 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\textsuperscript{61} and locus type, developmental stage and group (*F.
polyctena* –like or *F. aquilonia*–like) as explanatory variables and locus\_ID as a
random factor.

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Author contributions

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 availability

Data will be deposited into Dryad upon acceptance.

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