Differences in Thermal Tolerance between Parental Species Could Fuel Thermal Adaptation in Hybrid Wood Ants

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Differences in thermal tolerance between parental species could fuel thermal adaptation in hybrid wood ants

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Abstract

Genetic variability is essential for adaptation and could be acquired via hybridization with a closely related lineage. We use ants to investigate thermal adaptation and the link between temperature and genetic variation arising from hybridization. We test for differences in cold and heat tolerance between Finnish *Formica polyctena* and *Formica aquilonia* wood ants and their naturally occurring hybrids. Using workers, we find the parental individuals differ in both cold and heat tolerances and express thermal limits which reflect their global distributions. Hybrids however cannot combine thermal tolerance of parental species as they are equally heat-tolerant to *F. polyctena*, but not equally cold-tolerant to *F. aquilonia*. We then focus on a single hybrid population to investigate the relationship between temperature variation and genetic variation across 16 years using reproductive individuals. Based on the thermal tolerance results, we expected the frequency of putative *F. polyctena* alleles to increase in warm years and *F. aquilonia* alleles to increase in cold years. We find support for this in hybrid males but not in hybrid females. These results contribute to understanding the outcomes of hybridization, that may be sex-specific or depend on the environment. Furthermore, genetic variability resulting from hybridization could help hybrid wood ants cope with changing thermal conditions.

Introduction

Today, numerous species face the effects of human-mediated disturbances to natural habitats. Ongoing anthropogenic-driven climate change requires local species to migrate or adapt to altering conditions to avoid possible extinction (Scheffers et al., 2016). Adapting to new conditions requires an adequate level of genetic variation in the population (Hoffman and Sgrò, 2011). If not present in the population (i.e., standing genetic variation), genetic variation can arise from de novo mutations (Barrett and Schluter, 2008; Orr, 2005) and/or through hybridization (Harrison and Larson, 2014). As with standing variation, hybridization with a related lineage can provide adaptive variation more quickly than de novo mutations (Hedrick, 2013).

Hybridization is widespread across taxa in both plants and animals (Dowling and Secor, 1997; McVay, Hipp and Manos, 2017; Rieseberg and Wendel, 1993; Slager et al., 2020; Taylor and Larson, 2019) and it is predicted to further increase due to climate change as species expand their ranges and new contact zones between closely related taxa are formed (Chunco, 2014). Hybridization has contributed to adaptation in the past (e.g. Heliconius Genome Consortium, 2012; Meier et al., 2017), and in some cases it has helped coping with new thermal regimes (Martins et al., 2019; Pereira, Barreto and Burton, 2014). However, hybridization can also lead to negative fitness consequences and break-down of coadapted gene complexes (Dobzhansky, 1936; Dobzhansky and Pavlovsky, 1958; Muller, 1942; Brideau et al., 2006). Loss of fitness in hybrids can be severe resulting in inviability or sterility, but it can also be more subtle, resulting in poor performance (Barreto, Pereira and Burton, 2015; McQuillan et al., 2018). However, as the environment changes, so can selection acting on hybrid genotypes. Yet, only few studies address the consequences of hybridization through time or in different environments (but see Darwin's finches; Grant, 2002 and desert sunflowers; Rieseberg et al., 2007).

Increasing global surface temperature is one of the most pressing human-mediated environmental disturbances that populations encounter in their habitat (Lenoir and Svenning, 2015; Root et al., 2003; Sala et al., 2000). Changes in thermal regime have already led to considerable range shifts in many species and taxonomic groups (Devictor et al., 2008; Jump et al., 2009; Scheffers et al., 2016; Wilson et al., 2005). Understanding the limits of thermal tolerance helps to assess species' vulnerability to environmental change. Furthermore, a question of current interest, is to understand to what extent hybridization could help populations to adapt to new temperatures in the face of climate change.

Wood ants and other Hymenoptera are excellent models for the study of hybridization because of their haplodiploidy. Males have a haploid genome and develop from unfertilized eggs while females (queens and workers) are diploid and arise through sexual reproduction (see Figure 1B). Consequently, recessive incompatibilities can be masked when heterozygous in females because of diploidy, while they will be revealed in haploid males, where they can lead to hybrid breakdown (Koevoets and Beukeboom, 2009; Kulmuni et al., 2010; Kulmuni and Pamilo, 2014; Beukeboom et al., 2015). Similarly, recessive beneficial alleles can be selected for in haploid males more efficiently. Because wood ant nests are located in the same place over several tens of years (Keller and Genoud, 1997), these organisms additionally provide a unique opportunity to examine the effects of hybridization over time.

In southern Finland, the two wood ant species *Formica polyctena* and *Formica aquilonia* naturally hybridize and form several hybrid populations (Beresford et al., 2017), one of which has been studied in detail over multiple years. This hybrid population, named Långholmen, encompasses more than 25 ant nests and harbours two distinct genetic lineages (named R and W), both of which have a hybrid origin (Kulmuni et al., 2020; Kulmuni and Pamilo, 2014; Kulmuni et al., 2010). Kulmuni and Pamilo (2014) have shown using microsatellite markers that gene flow has occurred between hybrid lineages, and that several

alleles introgressed between the lineages are under selection, especially in haploid males. Interestingly, while in the initial study introgressed alleles were selected against in males of the W lineage (Kulmuni and Pamilo, 2014), in a more recent study these alleles were detected at intermediate to high frequencies in adult males from the same lineage (Kulmuni et al., 2020). Moreover, SNP markers showed selection favouring introgressed alleles over development in W males (Kulmuni et al., 2020). These observations raise the question: why is the frequency of introgressed alleles significantly different between males sampled on different years? One hypothesis raised in Kulmuni et al. (2020) was that the fitness of the hybrids could depend on the environment. The two parental species have distinct distributions: *F. aquilonia* has a more northern distribution and can be found in Northern and Central Europe while *F. polyctena* has a more southern distribution from Central Europe to southern parts of Fennoscandia (Figure 1A). These different distributions could mean that parental species are adapted to different thermal regimes, in which case temperature is a candidate variable affecting the frequency of parental alleles in hybrids and potentially hybrid fitness.

In this study we first investigate the thermal tolerance of Finnish *F. aquilonia, F. polyctena* and their hybrids, and ask if they differ in their thermal regimes. If thermal tolerance differs between parental species, then hybridization could provide populations with genetic variation in thermal tolerance and potential to adapt to different thermal regimes. To investigate this idea, we ask if allele frequency changes correlate with temperature across multiple years in the Långholmen hybrid population, focusing on introgressed variation. Significant correlation between yearly temperature and introgressed variation suggests hybrid fitness may be environment-dependent. More broadly, it would be consistent with the hypothesis that genetic variation arising through hybridization could help populations adapt to

new or fluctuating temperature regimes (e.g. Jones et al., 2018; Oziolor et al., 2019; Smukowski Heil et al., 2019).

Material and Methods

Thermal tolerance experiment

Expectations

We examined thermal tolerance of wood ants with two widely used indicators: heat-knockdown resistance and chill-coma recovery (Jørgensen et al., 2019, Teets et al., 2019, Angilletta et al., 2007). We predicted that parental species differ in their thermal tolerance: northernmost-distributed *F. aquilonia* should be more cold-tolerant, whereas southernmost-distributed *F. polyctena* should be adapted to warmer environments and be more heat-tolerant. We also test thermal tolerance of *F. aquilonia* x *F. polyctena* hybrids and ask whether hybridization between *F. aquilonia* and *F. polyctena* could be adaptive, allowing hybrid populations to combine thermal tolerance of both parental species. Alternatively, hybridization could lead to poor thermal tolerance due to hybrid defects, leading to narrower thermal tolerance in hybrids compared to parental species (Lamare et al., 2018).

Sampling and colony maintenance for thermal tolerance experiment

In order to study and compare their thermal tolerance, we collected samples from populations of the two parental species *Formica aquilonia* (Aq) and *Formica polyctena* (Pol) along with *F. aquilonia* x *F. polyctena* hybrids (Hyb) in May 2019. All populations sampled are found within latitudes of N 59°51'0" to N 60°34'0" and longitudes of E 19°54'0" to E 24°2'0". All populations are located between 13m and 129m from sea level. Both parental species are polygynous (having up to hundreds of queens within a mound or nest) and supercolonial, forming networks of interconnected mounds (Fig. 1B). The high number of reproductive

queens in each nest induces average relatedness of near zero between individuals within a single nest, hence samples from the same nest can experience the same environmental conditions but have a variable genetic background. One parental *F. polyctena* and one hybrid population was collected from the Åland Islands, whereas all remaining populations were situated in Southern Finland (Fig. 1A). The parental populations represent Finnish lineages of the species and are from here on referred as Finnish *F. aquilonia* and Finnish *F. polyctena*. Although we cannot rule out some past admixture in the parental localities, they do represent two ends of a genetic continuum (see below and Beresford et al., 2017). We chose adult workers for the experiment, since they can be collected in large numbers in each nest to reach sufficient statistical power. To avoid any potential population effect, we sampled three populations for each group (Aq, Pol, Hyb), and three nests per population (except for the Långholmen hybrid population, where we sampled five nests). Overall, we used a total of 10 populations and 29 nests in the experiments (10 *F. aquilonia*, 8 *F. polyctena* and 11 hybrid nests, see Results). Species identity of the nests was inferred based on previous genetic studies using microsatellite markers (Kulmuni et al., 2010; Beresford et al., 2017) and was confirmed later with a genotypic analysis (see below). After collection, the samples were transferred to artificial nests and kept under stable conditions at +20°C and a light cycle of 14h light / 10h dark. All nests were under the same conditions for twelve days until the onset of the experiments. The colonies were watered daily and fed with ca. 5ml standard ant diet of agar, eggs and honey (Bhatkar and Whitcomb, 1970).

Heat knock-down experiment

In order to determine a challenging temperature for the heat-knockdown resistance assay, we first estimated the critical thermal maximum (CTmax). CTmax can be described as a stressful temperature where individuals rapidly lose coordinated muscle control and no motility

response is detected (Huey et al., 1992). We randomly chose six ants from each group (Aq, Pol, Hyb) from twelve randomly picked nests (72 individuals overall; see Table S1) and sorted the ants individually on petri dishes (⌀60mm). We tested CTmax in a walk-in climate chamber with stable temperature. The CTmax experiment was repeated with three different room temperatures: 41 °C, 46 °C and 51 °C. We determined 51 °C to be CTmax, since all samples immobilized within the first 27 minutes of the experiment.

Ants were fed and watered in the evening before the experiment. Based on results from the CTmax experiment, room temperature of 46 °C was used in the heat-knockdown resistance experiment. Knockdown resistance is defined as the time required for insects to lose mobility at high temperatures (Huey et al., 1992). Random workers sampled from the top of each nest box were placed each on their own petri dish (diameter 38mm). We used ten workers from each nest in the experiment, except two hybrid nests FA12 and FA18 where we used fifteen workers, altogether 300 individuals (Table S1). Because of polygyny, relatedness is low among workers from the same nest. Petri dishes had water-saturated cotton wool for hydration. Dishes were organized on eight trays randomly, to minimize the effect of any potential temperature gradient in the climate chamber. Four observers scanned the dishes during the experiment and switched places between trays every 30 minutes to minimize observer bias. The time of immobilization was marked when ants did not respond to light tapping anymore (no movement). Tapping of the dishes was done every 10 minutes in a systematic manner. Since falling into a coma was a long process, individuals that were not moving but waved antennas or legs were also marked. Ants were observed drinking from the water-saturated cotton wool during the experiment. Observers checked room and surface temperatures every 10 minutes. Mean room temperature during the experiment was 46°C. The experiment lasted four hours, after which we collected all individuals and stored them in -20°C for later genotypic analysis.

Because size may influence thermal tolerance, six individuals per nest from the heat-knockdown experiment were weighed from each group (54 *F. aquilonia* individuals, 54 *F. polyctena* individuals and 66 hybrid individuals). Individuals were kept in a drying oven (50℃) for five hours, after which dry weight was measured individually on a laboratory scale with precision of 10^{-4}g.

Chill-coma recovery

We tested different temperatures and cold treatment durations in a preliminary experiment with 20 ants from 20 randomly picked nests. Finally, 25 minutes in -13°C degrees was chosen to be assayed in the chill-coma recovery experiment as individuals were observed recovering in ca. 10 minutes after being taken out of the climate chamber.

Ants were fed and watered in the evening before the experiment. Chill-coma recovery is defined as the time required for insects to recover from prolonged exposure to extremely low temperatures (Huey et al. 1992). Following the sample scheme of the heat-knockdown resistance assay, we assayed ten workers from each nest in the experiment, except two hybrid nests FA12 and FA18 where we used fifteen workers, altogether 300 individuals (Table S1). Chill-coma recovery was measured in two batches, both with 150 ants and carried out on the same day. Five workers were sampled from the top of each nest and placed each on their own petri dish (diameter 38mm). Dishes were sorted randomly on five trays, to minimize the effect of potential temperature gradient in the climate chamber. Trays were moved to a walk-in climate chamber with a stable temperature of -13 ± 2 °C and kept there for 25 minutes. After 22 minutes each dish was gently tilted so that all ants were on the left side of the dish when trays were taken out of the chamber. Trays were then moved to room temperature (23 °C). Chill-coma recovery was measured from the time when trays were taken out of the climate chamber until the ant woke up and walked over a line drawn across the middle of each dish.

This was considered indicative of recovery as ants are generally known to immediately start exploring their surroundings when recovered (Angilletta et al., 2007). For both batches the experiment was recorded on video (so that simultaneous recovery events could be recorded precisely) and recovery times were documented by a single observer afterwards. All trays were recorded for 30 minutes and ants that did not move over the line during that time period were considered not recovered.

Genetic assignment of samples used in thermal tolerance experiment

To verify the group identity of each nest (Aq, Pol or Hyb), we used microsatellite markers to genotype six individuals from each nest and studied genetic structure. DNA extraction was done from the samples used for dry weight measurements (i.e., only samples from the heat-knockdown experiment). The purification of total DNA was performed with DNeasy Tissue kits (Qiagen) following the manufacturer's spin-column protocol for insects. Samples were genotyped based on previous genetic studies using nine microsatellite markers (Fe7, Fe17, Fy3, Fe19, Fe13, Fy15, Fy12, Fl29, and Fy13) and PCR conditions determined in earlier studies (Kulmuni et al., 2010; Beresford et al., 2017). Total DNA was amplified with polymerase chain reactions (PCR) in Veriti 96-well Thermal Cycler (Applied Biosystems) with fluorescent labelling. Genotypes were resolved by capillary electrophoresis with a 3730 DNA Analyzer (Applied Biosystems) using 500 ROX size standard. Lastly, genotypes were scored with GENEMAPPER version 4.0 (Applied Biosystems). As genotypes were compared to samples genotyped in an earlier study on the same system (Beresford et al., 2017) we used three reference samples of known genotypes in every PCR reaction to control for variation potentially introduced by different PCR and DNA Analyzer machines. Thermal experiment data is deposited in the Dryad Digital Repository: https://doi.org/10.5061/dryad.m63xsj3zq (Nygård et al., 2021).

Nests were assigned to Finnish *F. aquilonia*, Finnish *F. polyctena* and hybrids based on the combination of a principal coordinates analysis (PCoA), the analysis of population structure (STRUCTURE) and prior assignments from the same populations, when available (Beresford et al., 2017). The PCoA was done with GENALEX (Peakall and Smouse, 2006) and included samples from a previous study (Beresford et al., 2017) to verify that the assignments corresponded to previously identified group status (Aq, Pol, Hyb). Finnish *F. polyctena* populations from Åland Islands had not been collected before, and therefore lacked previous genetic comparison. To identify population structure and to further verify the classification of samples into Aq, Pol and Hyb groups, genotypes were analysed with STRUCTURE 2.3.4 (Pritchard, Stephens and Donnelly, 2000) varying the number of genetic clusters (K) from 1 to 10. We used an admixture model with independent allele frequencies and ran five iterations for each K-value with burn-in of 200,000 and 1,000,000 MCMC replicates after burn-in. ΔK statistics (Evanno, Regnaut and Goudet, 2005) were calculated with STRUCTURE HARVESTER (Earl, 2012, Fig. S1) to detect the uppermost hierarchical level of structure in the data.

Statistical analysis of thermal tolerance data

Differences in heat-knockdown resistance and chill-coma recovery times between the groups (Aq, Pol, Hyb) were compared with Kaplan-Meier survival curves and Cox proportional hazards model from *survival* and *survminer* packages in R (R Core Team 2019, software v. 4.0.2, Therneau and Grambsch, 2000). Although, individuals sampled from the same nest can be considered independent samples (see above) we controlled for the possibility of correlated results between individuals from the same nests, using a robust sandwich estimate of variance in the survival analysis, following Angilletta et al. (2007). Differences in weights between groups (Aq, Pol, Hyb) was studied with a Linear Mixed Model, using the *lmer* function in the

lme4 package (Bates et al. 2015). *Individual dry weight* was used as a response variable and *group identity* as fixed factor, *nest of origin* was used as a random effect in the model. *P*-values were calculated using *t*-values assuming a normal distribution, the significance threshold being fixed at 5%. Because dry weight was measured from six individuals from the heat-knockdown resistance experiment, mean weight per nest was calculated to include dry weight as explanatory variable in the chill-coma analysis.

Correlation between temperature and genetic variation in a hybrid population

Expectations

If parental species differ in their thermal regimes, hybrids could harbour genetic variation that allows them to cope with varying temperatures. Over time, this could be seen as a correlation between temperature and genetic variation in the hybrid population. To test if there is a correlation between temperature and frequency of putative parental alleles in hybrids, we utilized a single population, for which we have long-term data. The Långholmen hybrid population has been monitored since 2004 and consists of two lineages (R and W), both of which are of hybrid origin. First, we ask if the frequency of R and W alleles in the population correlates with spring temperature over a 16-year sampling period using the same microsatellite loci and protocol as above. The microsatellite markers used in this study have previously shown the R lineage to be genetically slightly closer to *F. polyctena* and the W lineage closer to *F. aquilonia* (Beresford et al., 2017). Hence, we hypothesized R alleles at the microsatellite loci could be of *F. polyctena* origin (the southernmost distributed parental species) and favoured in a warm environment, leading to higher frequencies of R alleles in warm years. Conversely, the frequency of W alleles at the microsatellite loci should be higher

in cold years, because of putative *F. aquilonia* origin (the northernmost distributed parental species). Second, we ask if the allele frequency variation across years displays a sex-specific pattern. This could be expected due to the difference in ploidy between males and females. If selection acts on recessive alleles in haploid males but these are masked in heterozygous females, this would cause more frequency variation in haploid males compared to diploid females. Third, we ask if data from a single year is consistent with the hypothesis that temperature during development causes differential survival of hybrid males with different numbers of W and R alleles. This was addressed by testing if the frequencies of the parental R and W alleles were significantly different between early (egg) and late (adult) developmental stages during a warm year (+2 °C compared to the 1963-2020 average, see Fig. S2).

Samples

No substructure between nests was previously observed within a lineage in the Långholmen hybrids (see supplementary material from Kulmuni et al., 2020). Individuals from the same nest or from different nests within Långholmen can therefore be considered as equally independent samples. In these species, queens mate once in their life, can live at least 5 years and have overlapping generations (Keller and Genoud, 1997), therefore allelic combinations from a single queen can be subjected to selection over multiple years. Genotype data for adult individuals (males and new queens born on the sampling year) for the years 2004, 2008 and 2011 were obtained from Kulmuni et al. (2010) and Kulmuni and Pamilo (2014). In addition, males and new queens collected in 2014, 2018 and 2020 were genotyped for the present study (see Table S2 for all samples used in this study).

To test if temperature during development could cause differential survival of hybrid males with different parental alleles, we utilized data from a single warm year (see results) and genotyped 42 males at early (small larva) stage and 29 males at late (adult) stage (see

Table S3 for all samples used in this study). Males from the R lineage were not included because they were not found during sampling. The same microsatellite loci and protocol were used here as above in the thermal tolerance experiment. The final dataset after filtering low quality samples contained 686 individuals. Microsatellite data is deposited in the Dryad Digital Repository: https://doi.org/10.5061/dryad.v6wwpzgvh (Martin-Roy et al., 2021).

Statistical analyses

To verify the absence of nest effect on the genotype data, a principal component analysis (PCA, see Fig. S3) was performed to detect any nest substructure within a lineage using the *indpca* function from the *hierfstat* package (Goudet, 2005) in R (R Core Team 2019, software v. 4.0.2). The following analyses were performed for each lineage and sex separately. Allele frequencies were calculated using the *pop.freq* function of the *hierfstat* package and transformed using the arcsine square root transformation (see Table S4 and S5 for raw allele frequencies). The allele status (i.e. introgressed from R to W, introgressed from W to R, diagnostic for W, diagnostic for R or origin unknown) was determined following Kulmuni et al. (2010). Meteorological data from 2004 to 2020 were downloaded from the Finnish Meteorological Institute (https://en.ilmatieteenlaitos.fi, last accessed 03/10/2020), using observations from the Hanko Tvärminne meteorological station, located within a 500-meter radius of the study population. For each year, we used the mean overall temperature as well as the mean minimum and mean maximum temperatures in April to test for the correlation between temperature and allele frequencies, as this is the time when the majority of the new queens and males develop. We also considered the precipitation amount and snow cover depth as additional variables (see Table S6).

We asked if the change in allele frequencies between years correlates with temperature change between years. Allele frequency change was calculated by subtracting the arcsine-

square-root-transformed frequencies of a given year by the transformed frequencies of the previous year for which samples are available (see Table S2, i.e., frequency delta = frequency year (n+1) – frequency year (n)), and similarly for temperature change. Change in allele frequencies was used as a response variable in a Linear Mixed Model, using the *lmer* function in the *lme4* package (Bates et al. 2015) assuming a Gaussian distribution (Shapiro-Wilk Tests: *p*-values > 0.05). *Temperature delta* and *locus* were used as fixed factors in the model, *year* was used as a random effect and the model was weighted using the *allele count*. *P*-values were calculated using *t*-values assuming a normal distribution, the significance threshold being fixed at 5%. We report R^2 values for information only.

Finally, we utilized data from a warm year to test if the distribution of parental alleles per individual was different between early and late developmental stages in males. This result would be consistent with differential survival as a cause of fluctuations in male allele frequencies across years and call for more detailed studies to test the causative role of temperature for hybrid fitness in the future. To test for changes in the distribution of W diagnostic and alleles introgressed from R during development, we counted the number of these alleles present at the nine genotyped loci per single male in the W lineage (loci containing both W and R alleles were removed when analysing W diagnostic alleles changes), for both early (N = 42) and late (N = 29) developmental stages. Then, we performed a one-sided Wilcoxon rank sum test with continuity correction to estimate if the proportion of these alleles changed during male development according to our predictions (i.e. positive selection acting on R alleles and negative selection acting on W alleles, since it was a warm year).

Results

Thermal tolerance between wood ant species and their hybrids

Workers in *F. polyctena* and hybrids are bigger and are more heat-tolerant than in *F. aquilonia*

Genotypic analysis confirmed most of the initial classification of nest samples used in the thermal tolerance assays. Apart from the Sammatti population (for which a single nest was sampled in Beresford et al., 2017) and populations from Åland (which have not been studied previously), all the remaining populations retained their initial assignments (Table S1). Based on PCoA (Fig. S4) and STRUCTURE (Fig. S5) results, the Sammatti population was assigned as having one Finnish *F. aquilonia* nest and two Finnish *F. polyctena* nests and Jarsö (from Åland Islands) was assigned as a hybrid population. Reassignment of the nests did not affect the thermal tolerance results, since even after excluding Sammatti and Jarsö nests from the survival analysis, results remained significant.

There was a statistically significant difference in dry weights between groups (Aq, Pol, Hyb), Finnish *F. polyctena* and hybrids being bigger than Finnish *F. aquilonia*. Dry weight of the Finnish *F. aquilonia* (M=2.14 mg, SD=0.323) and Finnish *F. polyctena* (M=2.75 mg, SD=0.445) differed significantly (Estimate = - 0.6091; SD = 0.2203; *p*-value < 0.05). Hybrids (M = 2.66 mg, SD = 0.549) differed significantly (Estimate = -0.5679; SD = 0.2030; *p*-value < 0.05) from Finnish *F. aquilonia* but not from Finnish *F. polyctena* (Estimate = -0.0412; SD = 0.2158; *p*-value = 0.84). Individual dry weights ranged from 1.1 milligrams to 4.7 milligrams (Fig. 2A).

To study thermal tolerance in parental species and their hybrids, ten ants per nest and fifteen ants from hybrid nests FA12 and FA18 (N_{total}=300) were exposed to 46°C and their activity was recorded for four hours. Finnish *F. polyctena* individuals survived significantly longer (on average 17% longer) in the heat compared Finnish *F. aquilonia* (M±SD = 171±6.8 and 155±5.9 min for *F. polyctena* and *F. aquilonia*, respectively, *p*-value < 0.001, log-rank test, Fig. 2B). Hybrid ants survived longer than Finnish *F. aquilonia* individuals (M±SD =

191±5.3 min for hybrids). Hybrids seemed to slightly outperform Finnish *F. polyctena* individuals in terms of survival probability in the heat, but the difference was not significant (*p*-value = 0.054, log-rank test, Fig. 2B). There was considerable variability in survival between populations, but the differences between groups (Aq, Pol, Hyb) were not driven by a subset of populations.

Mean dry weight per nest had a highly significant positive effect on the survival probability (Cox proportional hazards model, *p*-value < 0.001, Wald $\chi^2_{1,298}$=17.44, Table S7). Therefore, bigger workers survived longer under heat conditions. In model comparison, the effect of the mean dry weight per nest remained significant even after species status was accounted for (*p*-value < 0.007, ANOVA), suggesting both species status and weight explain variation in thermal tolerance. The interaction term between mean weight and the species status was not a significant addition to the model (*p*-value = 0.75, ANOVA). The effect of weight on survival in the heat remained significant in both cases; either using individual weights and performing the analysis for a subset of individuals, for which weight was measured (N=175), or by using the mean weight per nest and all samples (N=300).

F. aquilonia **is more cold-tolerant than** ***F. polyctena*** **or the hybrids**

To test whether Finnish *F. aquilonia* individuals would be more cold-tolerant than Finnish *F. polyctena* individuals, ten ants from each nest were exposed to -13°C for 25 minutes in the chill-coma recovery experiment (N_{total}=300). Finnish *F. aquilonia* ants started to recover faster than Finnish *F. polyctena* ants (M±SD = 13.5±0.9 and 16.4±1.1 min, respectively), survival rates differing significantly between them (*p*-value < 0.001, log-rank test, Fig. 2C). Hybrid ants recovered slower (M±SD = 17.0±0.9 for the hybrids) than Finnish *F. aquilonia* individuals (*p*-value < 0.001, log-rank test, Fig. 2C) but had no difference in survival probability compared to Finnish *F. polyctena* ants (*p*-value = 0.67, log-rank test, Fig. 2C).

Recovery time from chill-coma ranged from 6.6 to 28 minutes. Overall, 93 ants (46 hybrids, 21 *F. aquilonia* and 26 *F. polyctena*) did not wake up during the monitoring time of 30 minutes after cold treatment. Chill-coma recovery experiment was carried out in two consecutive trials, but there was no batch effect on the recovery of the individuals (*p*-value = 0.43, log-rank test). There was considerable variability in the survival between populations also in the chill-coma recovery experiment, but as for the heat knockdown resistance experiment, the differences between groups were not driven by a subset of populations. Mean weight per nest did not have a significant effect on the recovery from cold treatment (*p*-value = 0.112, Wald $\chi^2_{1,300}$= 2.53, Table S8).

Correlation between temperature and genetic variation in a hybrid population

Alleles putatively of *F. aquilonia* origin have higher frequencies in hybrid males on colder years

We inspected correlation between genetic variation and temperature in one hybrid population with long-term genetic data. The hybrid population consists of two hybrid lineages, W and R. Results of the thermal tolerance assays showed *F. aquilonia* to be more cold-tolerant than *F. polyctena*. Consequently, we hypothesized alleles of *F. aquilonia* origin, namely W alleles, to be "cold" adapted and correlate negatively with temperature. In other words, we expected that an increase in temperature in April from one sampling year to the next would be coupled with a significant decrease in frequencies of the W diagnostic alleles in males of the W lineage. This hypothesis is supported when considering the mean minimum temperature in April (Estimate = -0.1049; SD = 0.0412; *p*-value < 0.05, see Table S6) but not in the case of overall mean temperature (Fig. 3C and 3A). Absolute allele frequency fluctuations are presented in

Supplementary Figure 6. We also hypothesized that changes in allele frequencies could differ between sexes possibly due to ploidy. This was supported as for females, no significant correlation between change in allele frequencies and change in temperature of both *F. aquilonia*-like diagnostic and introgressed alleles was found (Fig. 3B and 3D).

Alleles putatively of *F. polyctena* origin have higher frequencies in hybrid males on warmer years

We hypothesized R alleles to be putatively of *F. polyctena* origin and potentially warm adapted. Hence, based on the thermal tolerance assays we expected R alleles to correlate positively with yearly temperature. Frequencies of the R diagnostic alleles in both R males and R females were stable across years independently of mean temperature shifts, a result that does not support our hypothesis (Fig. 4A and 4B). However, in males of the W lineage, when the mean temperature of April increased between years, alleles introgressed from the R lineage significantly increased in frequency (Estimate = 0.2370; SD = 0.0902; p-value < 0.01) (Fig. 4C), as expected under our hypothesis. Correlation between temperature and alleles introgressed from R was also significant in W males when considering the means of the minimum and maximum temperatures of April (see Table S6). In addition, frequencies of R lineage introgressed allele in W lineage females correlated significantly with the precipitation amount (see Table S6), however it is likely this result is an anomaly as precipitation amount was not an impacting meteorological factor in all other cases. Therefore, it is not considered in the discussion.

Differential survival of W males during a warm year: selection favours individuals with more R and less W alleles

The above results for males follow our expectations, namely that W alleles have higher frequencies at colder temperature and R alleles have higher frequencies at warmer temperatures. To test if correlation between temperature and W and R alleles in hybrid males is due to differential survival of males depending on their degree of W and R alleles, we measured larva to adult survival within a single year. We genotyped larvae and adults for the year 2014, which was a warm year (+2 °C compared to the 1963-2020 average, see Figure S2). Therefore, we expected selection against diagnostic W alleles (putatively cold adapted) but selection for R introgressed alleles (putatively warm adapted) in W males during development. Comparing allele distributions at early and late developmental stage revealed a significant decrease in the number of diagnostic W alleles present in males (Wilcoxon rank sum test score = 757; p-value < 0.05) and a significant increase of R introgressed alleles (Wilcoxon rank sum test score = 460.5; p-value = 0.025; Fig. 5), supporting our hypothesis for a warm year. These results remain significant for the R introgressed alleles (p-value < 0.05) and are not significant for diagnostic W alleles (p-value = 0.05) if we perform the analysis keeping only individuals sampled from nest FA17, suggesting sampling different nests does not drive these results. Note that the two loci containing the introgressed alleles (from one donor lineage) also have diagnostic alleles (from the other, recipient lineage), so under our hypothesis the increase in R alleles could cause the decrease of W alleles at the same loci.

Discussion

In order to survive, species and populations need to adapt to rapidly changing environments. Genetic variation within a population is essential for adaptation and it can be acquired through hybridization with a related lineage (Grant and Grant, 2019). Due to climate change, rates of hybridization are predicted to increase, but rather than being a threat to biodiversity,

hybridization could help populations adapt into new temperature regimes. Indeed, hybrids with parental species differing in thermal regimes could harbour genetic variation allowing them to cope with varying temperatures. This could cause differential survival of individuals with different parental alleles and over time, could be seen as a correlation between temperature and genetic variation in the hybrid population. Here we show that Finnish populations of two wood ant species, *F. aquilonia* and *F. polyctena*, differ in both heat and cold tolerance, while their hybrids show thermal tolerance close to *F. polyctena*. Based on these results we predicted that genetic variation within a hybrid population could be linked to temperature variation across years. Using long-term genetic data, we show that allele frequency changes at nine loci in males were correlated with changes in the temperature in April (i.e. during sexual larvae development) over a 16-year time period. Furthermore, and in agreement with these results, we found that during a warm year, the number of alleles from the Southernmost parental species increased during development in males, while those of the Northernmost parental species decreased. These results are consistent with the hypothesis that temperature variation (or something correlated with it) across multiple years could promote maintenance of (potentially adaptive) genetic diversity from both parental species within the hybrid population. Furthermore, hybrids could show adaptive potential in fluctuating temperatures. These results suggest that outcomes of hybridization can be context-dependent and vary in time.

Parental species differ in their thermal adaptation, and hybrid workers show similar thermal regime to *F. polyctena*

Finnish *F. polyctena* was more heat-tolerant and survived longest in the heat-knockdown resistance experiment, whereas Finnish *F. aquilonia* appeared to be more cold-tolerant and recovered fastest from the chill-coma recovery experiment. Our results suggest that thermal

tolerance in these wood ants is at least partly genetic (instead of plastic), because even when collected from a similar environment and latitude, the two species significantly differ in their thermal tolerance. Thermal tolerance limits are critical in the life cycle of ectothermic organisms. Studies have shown that these limits are often linked to geographic distributions of the species (Andersen et al., 2015; Kellerman et al., 2012; Sunday, Bates and Dulvy, 2012), minimizing the energetic cost of thermoregulation by adapting to local surroundings.

The hybrid nests could not combine thermal tolerance of their parental species even though they have alleles from both heat-tolerant *F. polyctena* and cold-tolerant *F. aquilonia*. Overall, the hybrids expressed thermal limits that were more similar to Finnish *F. polyctena* than to Finnish *F. aquilonia*, which is also consistent with the observation that hybrids appeared to be genetically closer to Finnish *F. polyctena* than to Finnish *F. aquilonia*. Thus, the similar responses observed between hybrids and Finnish *F. polyctena* ants could result from similarities in their genetic background. Thermal tolerance in hybrids has been tested across taxa and outcomes vary substantially (Culumber et al., 2012; Lockwood, Gupta and Scavotto, 2018; Martins et al., 2019; Wells et al., 2016). Hybrids can outperform both parental species (Pereira, Barreto and Burton 2014), do worse than either parent (Martins et al., 2019), match the heat-tolerant parental lineage (Lockwood, Gupta and Scavotto, 2018), or show no significant difference (Wells et al., 2016).

Our results suggest that heavier wood ants (i.e., with larger body size) can tolerate challenging heat conditions better than smaller individuals. Body size differed between the species, and on average Finnish *F. aquilonia* individuals were the smallest while hybrids and Finnish *F. polyctena* were the biggest. However, even when controlling for the species status (Aq, Pol or Hyb), body size explained a significant proportion of variation in survival in heat, suggesting

that both size and species status play a role in survival. We did not find a clear effect of body size on cold tolerance, which could be due to methodological issues, since mean weight per nest was calculated from the heat-knockdown experiment samples. However, other studies with several ant species have also shown no correlation between body size and chill-coma resistance (Maysov, 2014; Modlmeier et al., 2012). To verify the lack of such relationship in wood ants, future studies will require extensive measurements of individual weights also from the samples used in the cold tolerance experiments. Upper limits of thermal tolerance often increase with body size, as shown in species across the *Formicidae* family (Clémencet et al., 2010; Ribeiro, Camacho and Navas, 2012; Wendt and Verble-Pearson, 2016). Thermal tolerance of the workers is crucial for the colony's well-being, since workers are responsible for the mandatory tasks such as cleaning, foraging and taking care of the brood. Larger body size can result in, for example, more effective desiccation tolerance and cuticular thermal resistance (Cerdá and Retana, 1997; Galushko et al., 2005).

Sex-specific allele frequency variation maybe linked to temperature-dependent selection in a hybrid population

Our previous study documented significant genetic changes between samples collected 10 years apart in a single hybrid population (Kulmuni et al., 2020). Given the above results on different thermal tolerance between the parental species, we hypothesized that genetic changes in the hybrid population could be caused by temperature dependent selection due to variation in yearly temperature. Indeed, our results revealed significant correlation between allele frequency and temperature changes in the Långholmen hybrid population over 16-year time period that was sex-specific. In years when temperature during development is warmer, R alleles (putatively of *F. polyctena* origin) increase in frequency in adult W males compared to colder years. On the other hand, in years when temperature is colder, W alleles (putatively

of *F. aquilonia* origin) increase in frequency in adult W males compared to warmer years. These results are unlikely to be explained by migration outside the hybrid population as wood ant populations are genetically differentiated (Beresford et al., 2017) and we have not observed new microsatellite alleles entering the population during our study period. Furthermore, population samples collected from nature in 2014 revealed that in W males, W diagnostic alleles decreased significantly from early to late developmental stage while R introgressed alleles increased, as expected on a warm year such as 2014. This result is consistent with the idea that temperature or another variable correlated with temperature could drive allele frequency variation in males across years. However, under this hypothesis selection may not act independently on parental alleles of different origin as they partly occur at the same microsatellite loci. Furthermore, we do not assume that microsatellite alleles themselves are under selection, but they could be near the true genomic targets of selection.

We did not find a significant correlation between allele frequencies in females (i.e. new queens) and yearly temperature. Different results in males and females could be related to their ploidy difference. The fact that allele frequency fluctuations correlate with temperature in males but not in females could be due to recessive alleles: they are exposed to selection in the haploid males, making response to potential selection stronger in haploids compared to diploids (Beukeboom et al., 2015; Koevoets and Beukeboom, 2009; Nouhaud et al., 2020). On the other hand, diploidy allows females to carry both *F. polyctena* and *F. aquilonia* alleles simultaneously in heterozygotes, providing a sort of "fitness insurance". Heterozygous status in workers is beneficial if they could combine thermally-adapted traits from both parents. Heterozygosity in queens could be beneficial if it allows a proportion of their male offspring to survive every year despite temperature variations. This idea is consistent with results from

Kulmuni and Pamilo (2014), who showed that heterozygous females for introgressed alleles were indeed selected for in the Långholmen supercolony.

Given results from both of our experiments, studying thermal tolerance of both males and new queens is required in the future. Particularly, we would expect males to show more variation in their tolerance compared to females. Moreover, males with R alleles are expected to do better in heat compared to males with more W alleles. Future studies also need to investigate microclimatic temperature conditions near the nests and how it relates to within-nest temperature at the location where developing individuals are maintained, since these are likely to be the key thermal variables affecting survival. An earlier study on *F. polyctena* (Frouz and Finer, 2007) has demonstrated that outside temperature could impact within-nest temperature.

Maintenance of genetic polymorphisms in the *Formica* hybrids

In other taxa, fluctuating selection induced by the abiotic environment has been shown to impact genetic diversity via frequency change of alleles under selection (e.g. Bergland et al., 2014; Dahlhoff and Rank, 2007; Hornoy et al., 2015; Jump et al., 2006). However, the conditions under which genetic polymorphism can be maintained over long periods via temporally varying selection are still debated because many models require very strong selection (Bergland et al., 2014, but see Buffalo and Coop, 2020). Recent theoretical studies have outlined biologically realistic scenarios for the maintenance of polymorphisms (Bertram and Masel, 2019; Park and Kim, 2019; Wittmann et al., 2017) several of which are compatible with the ant system. The first mechanism is the storage effect, where a portion of the population is protected from selection because of a protective life history trait (Chesson, 1985; Chesson and Warner, 1981). Indeed, the reproductive queens are buried deep in the ground in the warmth of the nest as they do not forage and hence are buffered from

fluctuating outside temperatures. The storage effect requires overlapping generations and strong selection to maintain long-term genetic polymorphism (Park and Kim, 2019), both compatible with the ant system. Queens are long-lived and reproduce over multiple years, allowing them to potentially produce offspring of similar genetic background independently of the temperature. Furthermore, every year a huge surplus of eggs is produced as there are hundreds of queens per nest and each queen can lay up to 100 eggs per day over multiple days. This means the population can sustain very strong mortality (i.e. selection) during egg development. In addition, genetic mechanisms such as cumulative overdominance (i.e. incomplete dominance reducing selection against rare alleles) and reversal of dominance (i.e. dominance changes over time so that the favoured allele at any moment is dominant) could maintain genetic polymorphism according to theory (Bertram and Masel, 2019; Wittmann et al., 2017). We do not know if these mechanisms are at play in the ant system, but haplodiploidy does influence dynamics of recessive alleles as described above and could influence the maintenance of polymorphisms (Ghenu et al., 2018). In summary, many ecological and genetic mechanisms could promote maintenance of balanced polymorphisms, especially when acting conjointly (Bertram and Masel, 2019). However, further studies are needed to investigate genome-wide patterns of polymorphisms and their maintenance in the ant system.

Future of the parental species and hybrids in warmer temperatures

In the future, the thermal regime of Northern Europe is likely to shift towards increasing overall temperatures, increased precipitation and snowless winters (Füssel et al., 2017; IPCC, 2014). Periods of intense heat are likely to increase while extreme cold periods become less frequent (Coumou and Robinson, 2013; Lorenz, Stalhandske and Fischer, 2019). Change in these thermal conditions is likely to expand species ranges poleward and to higher elevations

(Chen et al., 2011; Crickenberger and Wethey, 2018; Hickling et al., 2006; Pinsky et al., 2013). Considering the two species in this study and their hybrids, heat-tolerant *F. polyctena* may possibly extend its range northwards in the future, which could result in increased rates of hybridization. This may result in *F. aquilonia* becoming outcompeted in some regions and being restricted to shrinking Northern areas. If there will be more extreme temperature events, the hybrids could be advantaged, since they could be able to produce both types of males, one type thriving on a cold year and another type on a warm year. Albeit sparse, genetic data suggests pure *F. polyctena* populations are in minority in Finland (Beresford et al., 2017), and hybridization could have allowed them to colonize this far North in the first place, as in other taxa such as wasp spiders (e.g. Krehenwinkel and Tautz, 2013). Indeed, new genetic combinations created by hybridization allows for novelties and colonizing new niches (e.g. Rieseberg et al., 2007).

Conclusions

Due to climate change, rates of hybridization are predicted to increase, but rather than being a threat, hybridization could help populations adapt into new temperature regimes. Our results showed that the Finnish populations of *F. aquilonia* and *F. polyctena* have different thermal tolerances, reflecting their distributions in Europe. Hybrid female workers sampled from several populations could not combine thermal regimes of the parental species. Still, we find significant correlation in the frequencies of the parental alleles and temperature over years in hybrid haploid males, which we suggest is due to differential survival of males with different genotypes. Long-term studies on the outcome of hybridization events are rare (e.g. Rieseberg et al., 2007; Grant, 2002). However, *Formica* ants offer here a unique insight into evolutionary mechanisms taking place in hybrid populations. Haplodiploidy allows the detection of selection on recessive alleles that would be otherwise very hard to detect in

diploid models (if not located on sex chromosomes). Furthermore, polygyny can buffer the consequences of deleterious introgression, because the high number of reproductive queens will ensure colony survival despite some queens producing unfit offspring. In contrast to studies showing a link between temperature and genetic variation within a species, our study is novel in that we document this pattern in the hybrids. Wood ants are keystone species in the boreal forests and important for healthy forest ecosystems, but they are threatened by increasing global temperatures and habitat destruction. Our findings may help to predict the future of the wood ant populations in the face of global climate change.

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Author contributions: EN, PN, JK designed the thermal tolerance experiment. JK developed experimental design for correlation between temperature and genetic variation. RMR, EN, PN, JK collected samples. RMR, EN, PN, JK performed the thermal tolerance experiments. RMR and EN performed laboratory work and analysed the data. EN and RMR drafted original drafts and JK combined them. PN and JK supervised the work and reviewed & edited the manuscript. All authors approved the final version of the manuscript.

Data accessibility: Thermal experiment data is available at Dryad (Nygård et al., 2021, https://doi.org/10.5061/dryad.m63xsj3zq). Microsatellite data is available at Dryad (Martin-Roy et al., 2021, https://doi.org/10.5061/dryad.v6wwpzgvh).

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Figure legends

Figure 1: (A) Map of *F. aquilonia* (green) and *F. polyctena* (yellow) ranges. Overlapping ranges between the two species are represented in orange (adapted from Stockan and Robinson, 2016). The insert displays populations sampled in southern Finland and Åland for the present study, where the colour of each population indicates the species identity (green: *F. aquilonia*, yellow: *F. polyctena*, orange: hybrid). Note that nests from the Sammatti population were assigned to both parental species (see Results). (B) Life cycle of mound-building wood ants in our study system. Mated queens lay eggs during spring and summer every year. Eggs laid in the spring will develop into new queens and males. Eggs laid in the summer will develop into workers. Both queens and workers develop from fertilized eggs and are diploid. Males develop from unfertilized eggs and are haploid. New queens and males form a pool of reproductives, which can mate on top of their maternal nest or disperse and mate on another nest in the supercolony. The degree of long-distance dispersal is unknown, but presumably also happens sometimes. Queens mate only once in their life, usually with only a single male and can live at least 5 years while males die after mating. Each nest contains up to hundreds of mated queens with overlapping generations. Mated queens can also establish new nests near their maternal nest. Workers and resources can be exchanged between nests within the supercolony.

Figure 2. (A) Distribution of individual weights among three groups: Finnish *F. aquilonia* shown in green, Finnish *F. polyctena* in yellow and hybrids in orange. (B) Kaplan-Meier survival curves from the heat-knockdown resistance experiment. The x-axis indicates the time in minutes and the y-axis the percentage of surviving individuals (95% confidence intervals are indicated with lighter colours). The Finnish *F. aquilonia* group has the steepest curve and individuals start to fall into heat-coma significantly quicker than hybrids or *F. polyctena*, for which no significant difference was detected. (C) Kaplan-Meier survival curves from the chill-coma recovery experiment. The x-axis indicates the recovery time and the y-axis the cumulative hazard, i.e. the accumulation of recoveries over time. 95% confidence intervals are indicated with lighter colours. *F. aquilonia* has the steepest curve and individuals start to recover from chill-coma significantly quicker. Hybrids and *F. polyctena* recover slower and there are no significant differences in their recovery.

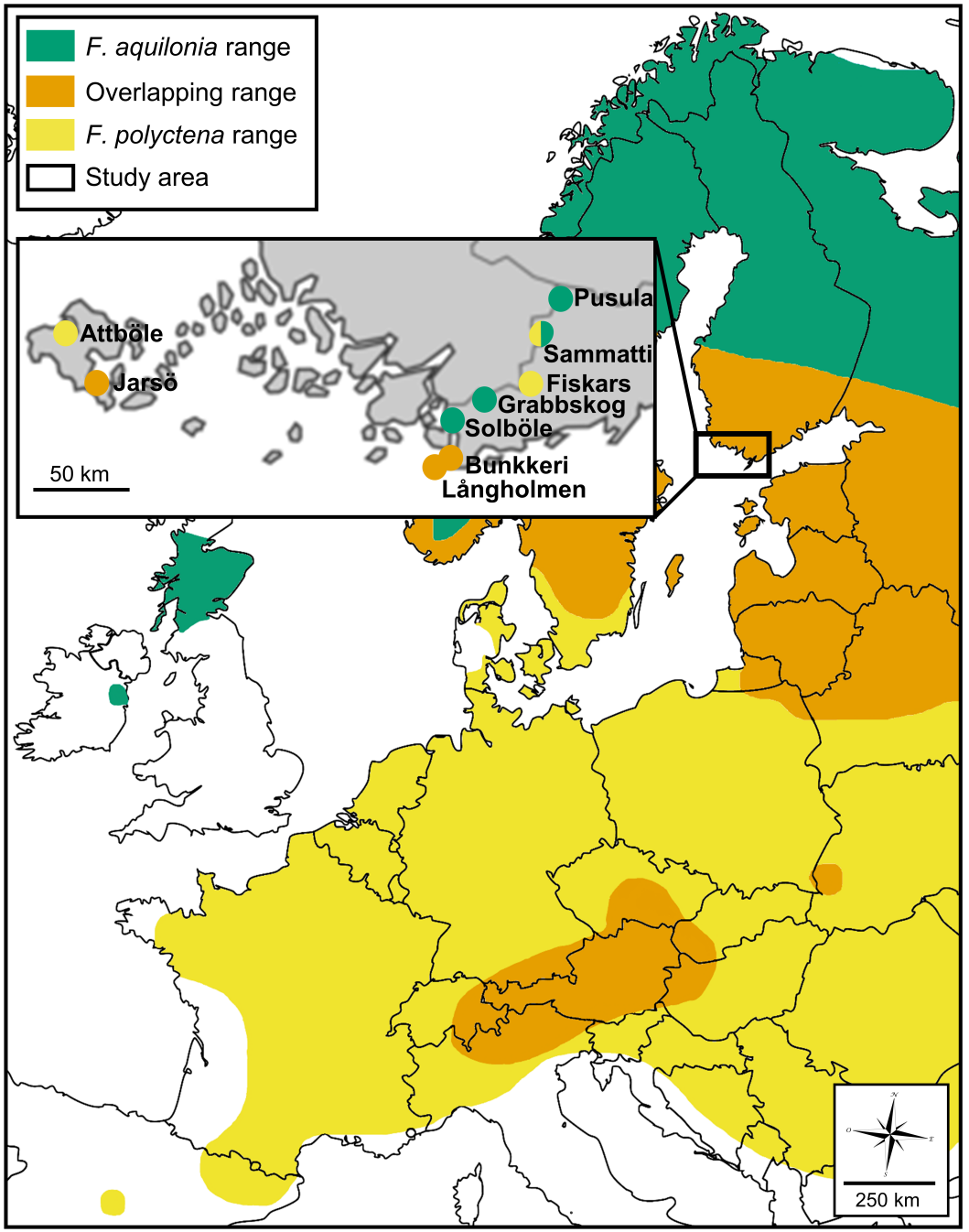
Figure 3: Year-to-year change in the frequency of W alleles (frequency delta) and change in mean temperature (minimum temperature in panel C) in April (temperature delta). Panel A shows alleles diagnostic to W lineage in

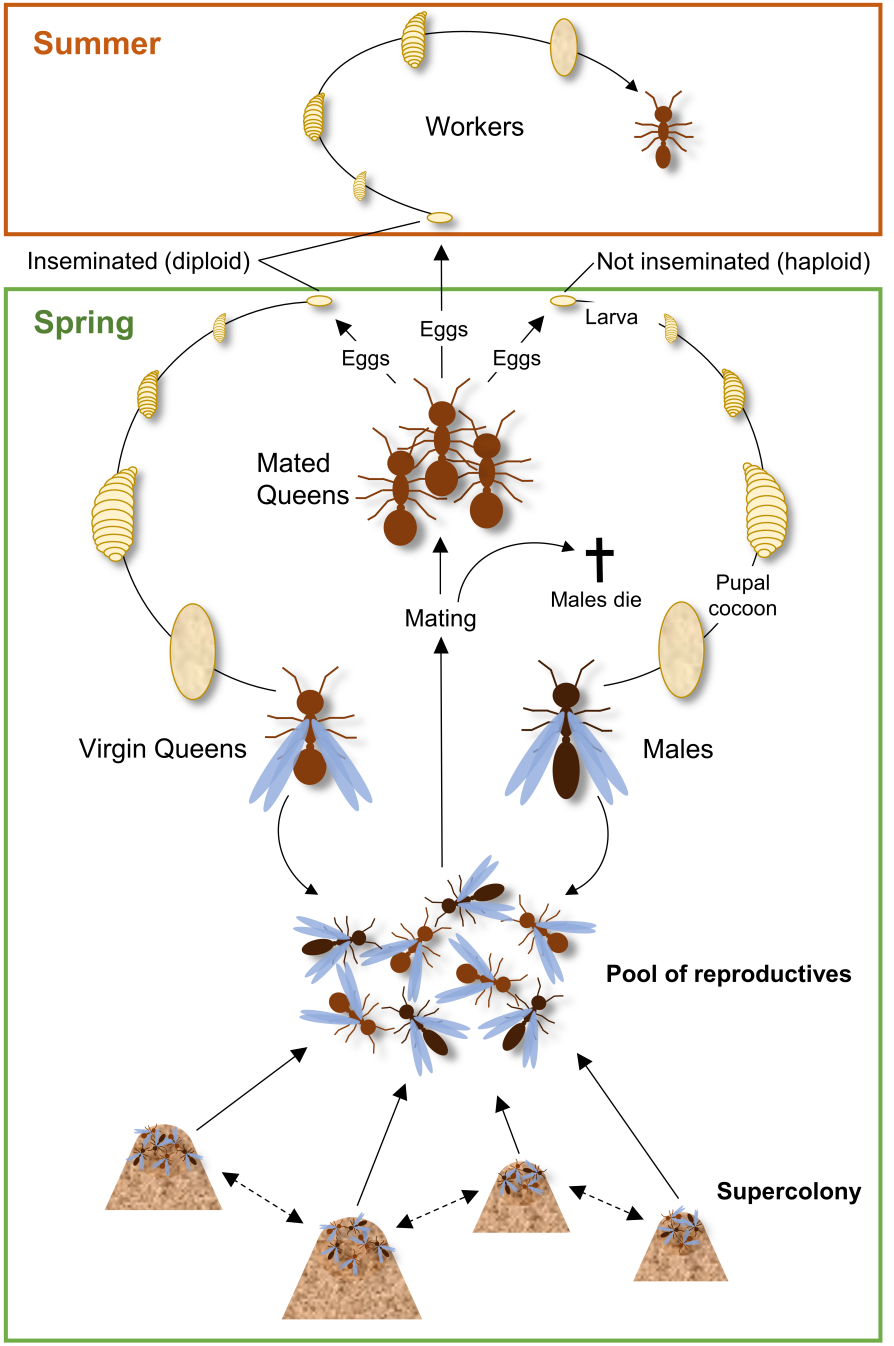
W males, B shows alleles diagnostic to W lineage in W females and panel D shows W alleles introgressed to R lineage females. R males are not shown here because they never possess alleles introgressed from the W lineage as adults, suggesting strong selection (Kulmuni and Pamilo, 2014). Panel A and C show the same data (i.e. W alleles diagnostic to the W males) but plotted using the mean temperature in April (Panel A) and mean minimum temperature in April (Panel C). Each allele is represented by a coloured point and a single allele is displayed per locus (see legend). Each data point in the figure represents change in allele frequency against change in April mean temperature from year n to year n+1 (i.e. delta; see Table S2 for years and individuals sampled). Note that samples do not come from exactly the same years for each sex and lineage, meaning all panels do not have the same number of datapoints. *P*-values were calculated using the Linear Mixed Model results. R^2 values are provided for information only.

Figure 4: Year-to-year change in the frequency of R alleles (frequency delta) and change in mean temperature in April (temperature delta) for males and females of both lineages. Each allele at a locus is represented by a coloured point. Each data point in the figure represents change in allele frequency against change in April mean temperature from year n to year n+1 (i.e. delta; see Table S2 for years sampled). Note that samples do not come from exactly the same years for each sex and lineage, meaning all panels do not have the same number of datapoints. *P*-values were calculated using the Linear Mixed Model results. R^2 values are provided for information only.

Figure 5: Change in the genetic composition of males from early developmental stage to late developmental stage in a single season inferred from a population sample. Proportion of males from the W lineage carrying *N* (from 0 up to 2) W diagnostic and/or R introgressed alleles at the 9 genotyped microsatellite loci, for both early (N = 42) and late (N = 29) developmental stages, for the year 2014.

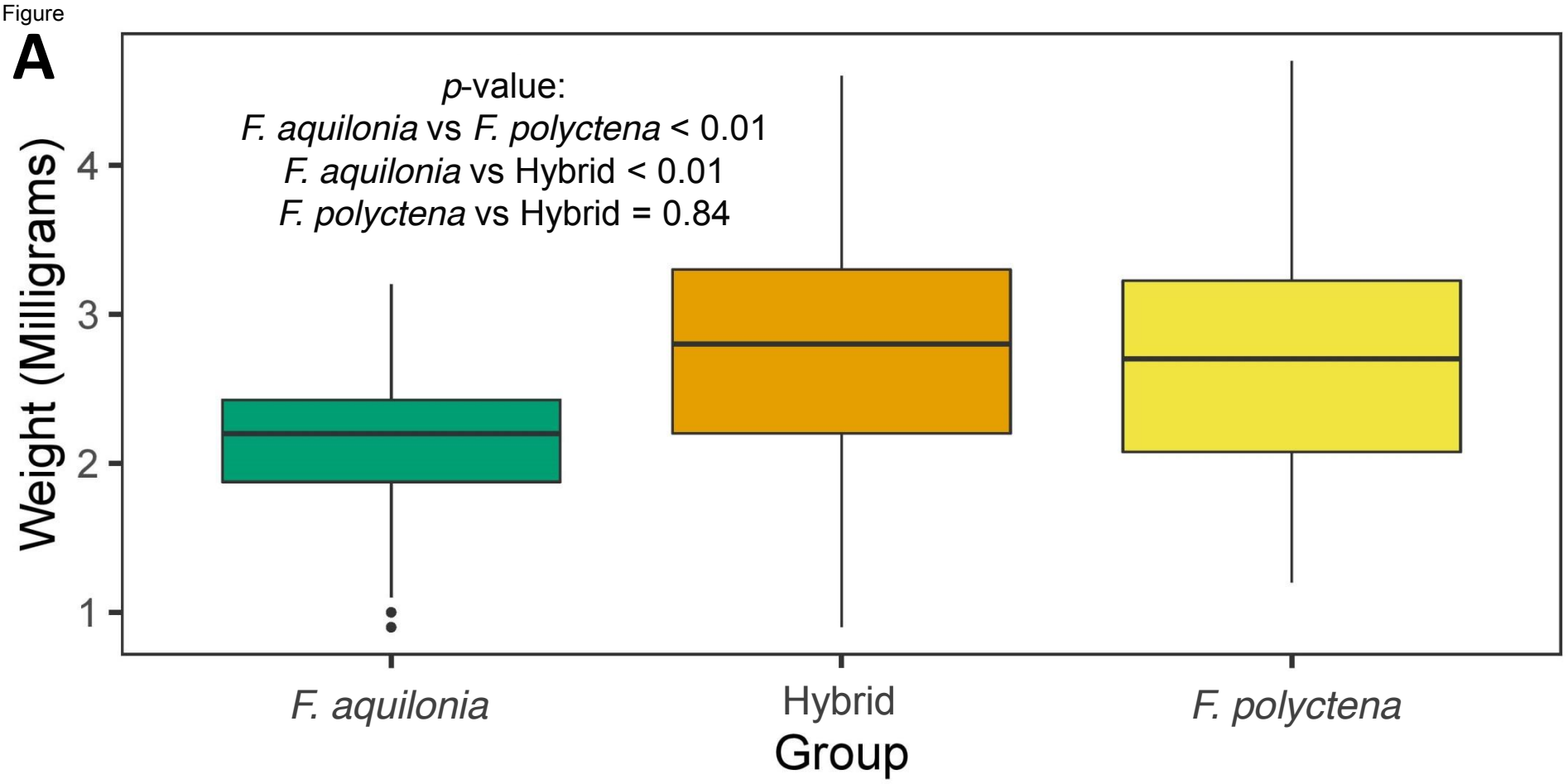
Figure


A
F. aquilonia range
Overlapping range
F. polyctena range
Study area
Pusula
Attböle
Sammatti
Jarsö
Fiskars
Grabbskog
Solböle
Bunkkeri
Långholmen
50 km
250 km

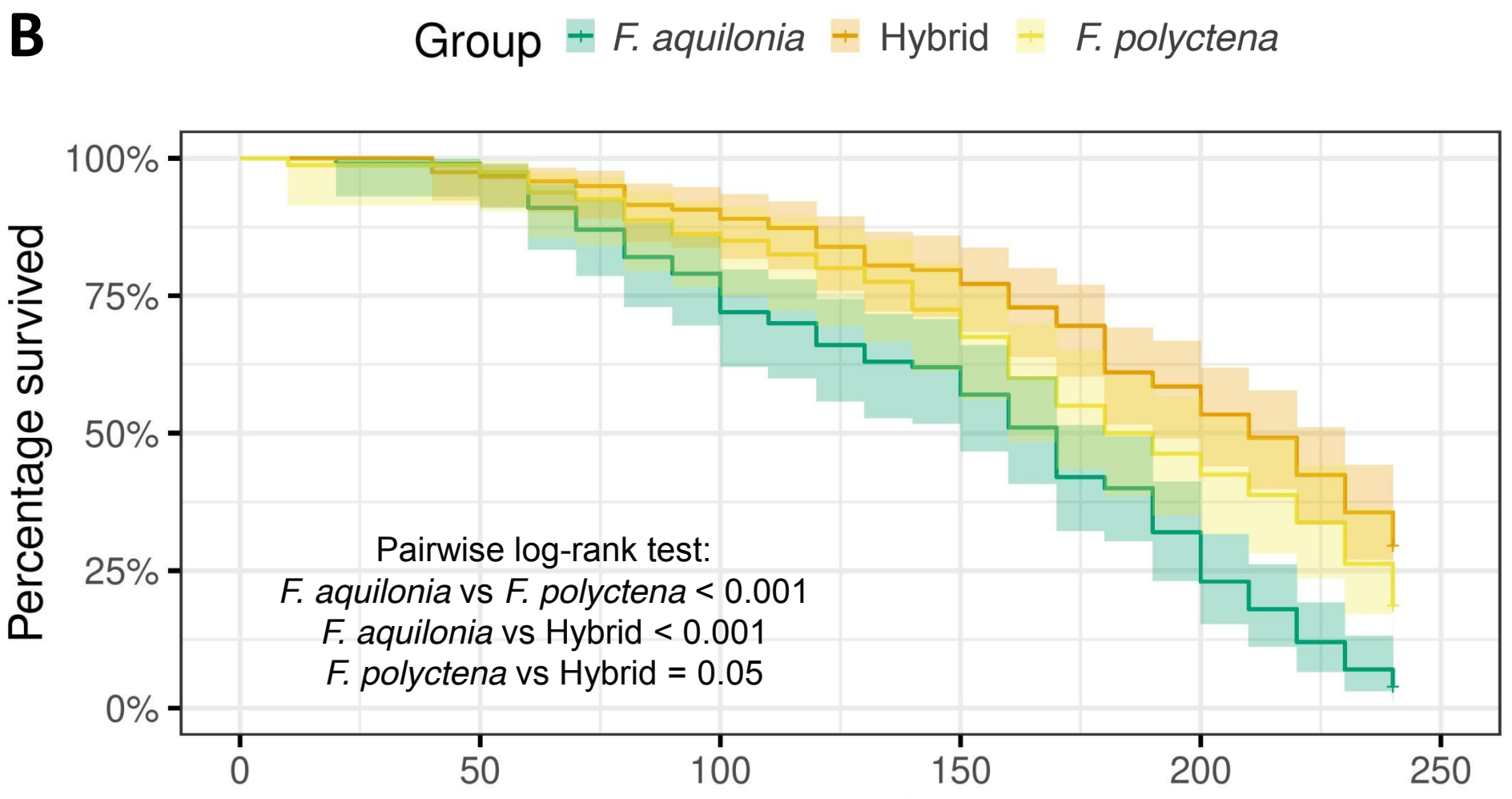

B
Summer
Workers
Inseminated (diploid)
Not inseminated (haploid)
Spring
Eggs
Eggs
Eggs
Larva
Mated Queens
Mating
Males die
Pupal cocoon
Virgin Queens
Males
Pool of reproductives
Supercolony

Figure

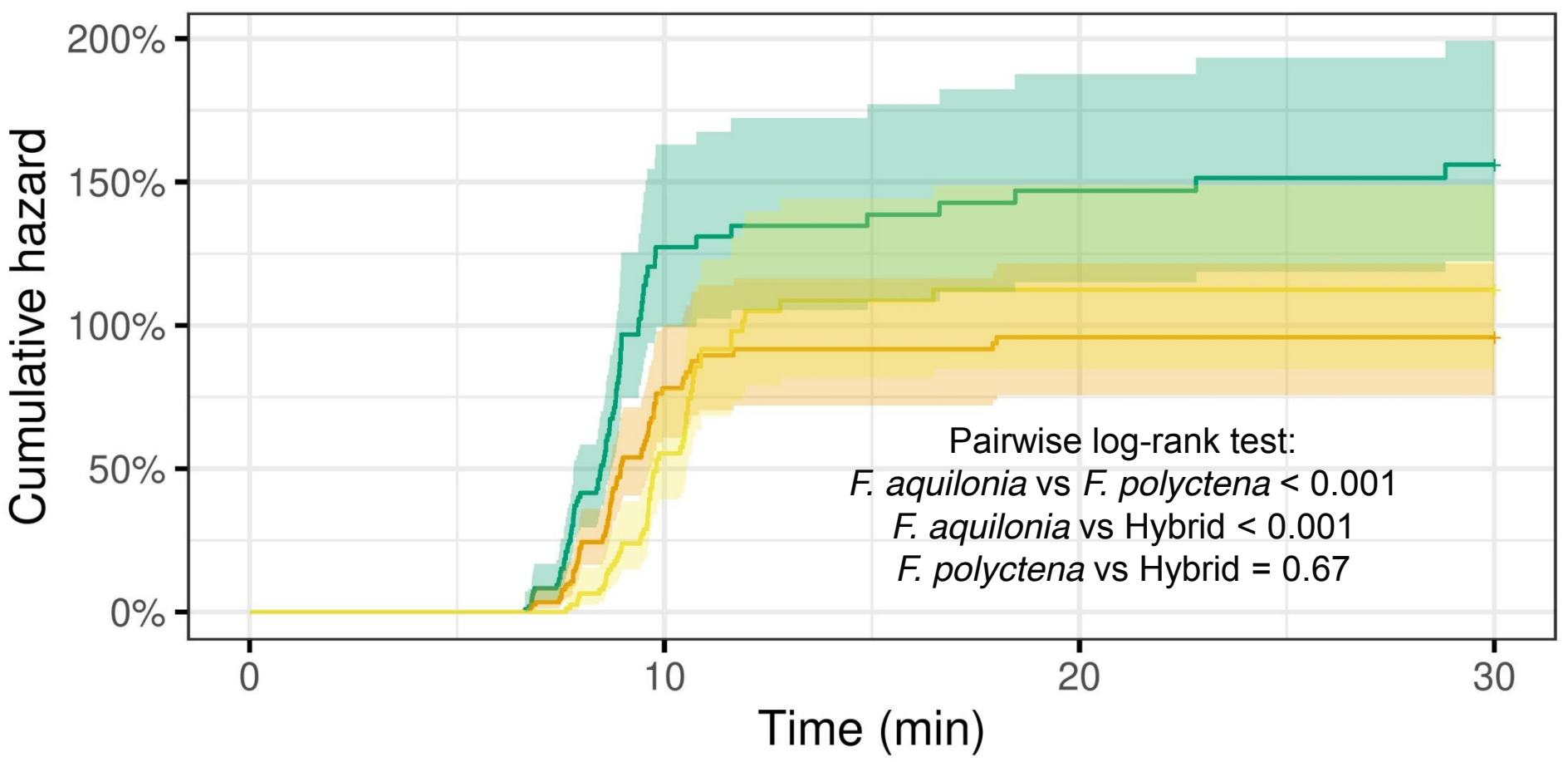
A


p-value:
F. aquilonia vs F. polyctena < 0.01
F. aquilonia vs Hybrid < 0.01
F. polyctena vs Hybrid = 0.84
Weight (Milligrams)
4
3
2
1
F. aquilonia
Hybrid
F. polyctena
Group

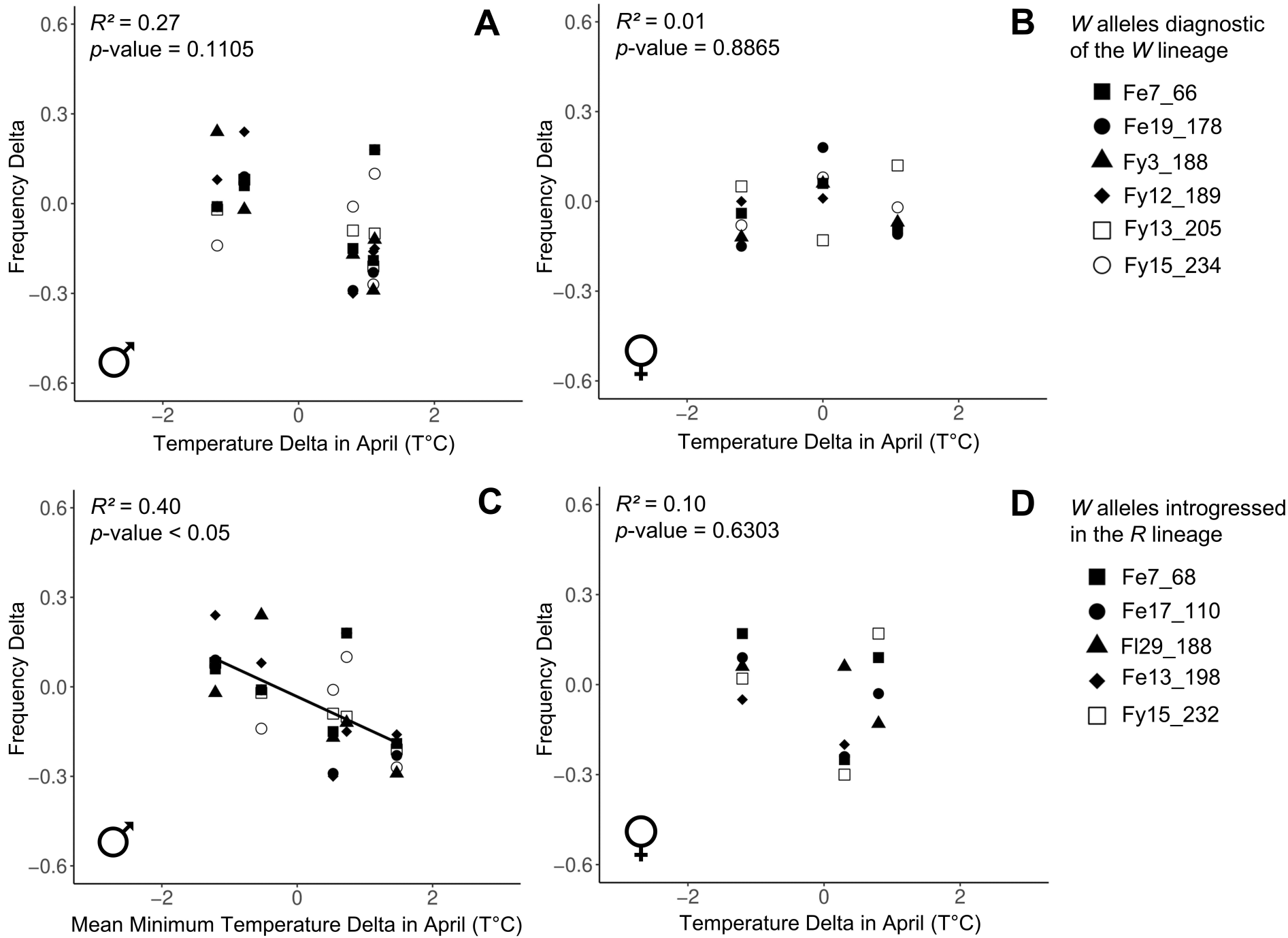
B


Group
F. aquilonia
Hybrid
F. polyctena
Percentage survived
100%
75%
50%
25%
0%
Pairwise log-rank test:
F. aquilonia vs F. polyctena < 0.001
F. aquilonia vs Hybrid < 0.001
F. polyctena vs Hybrid = 0.05
0
50
100
150
200
250
Time (min)

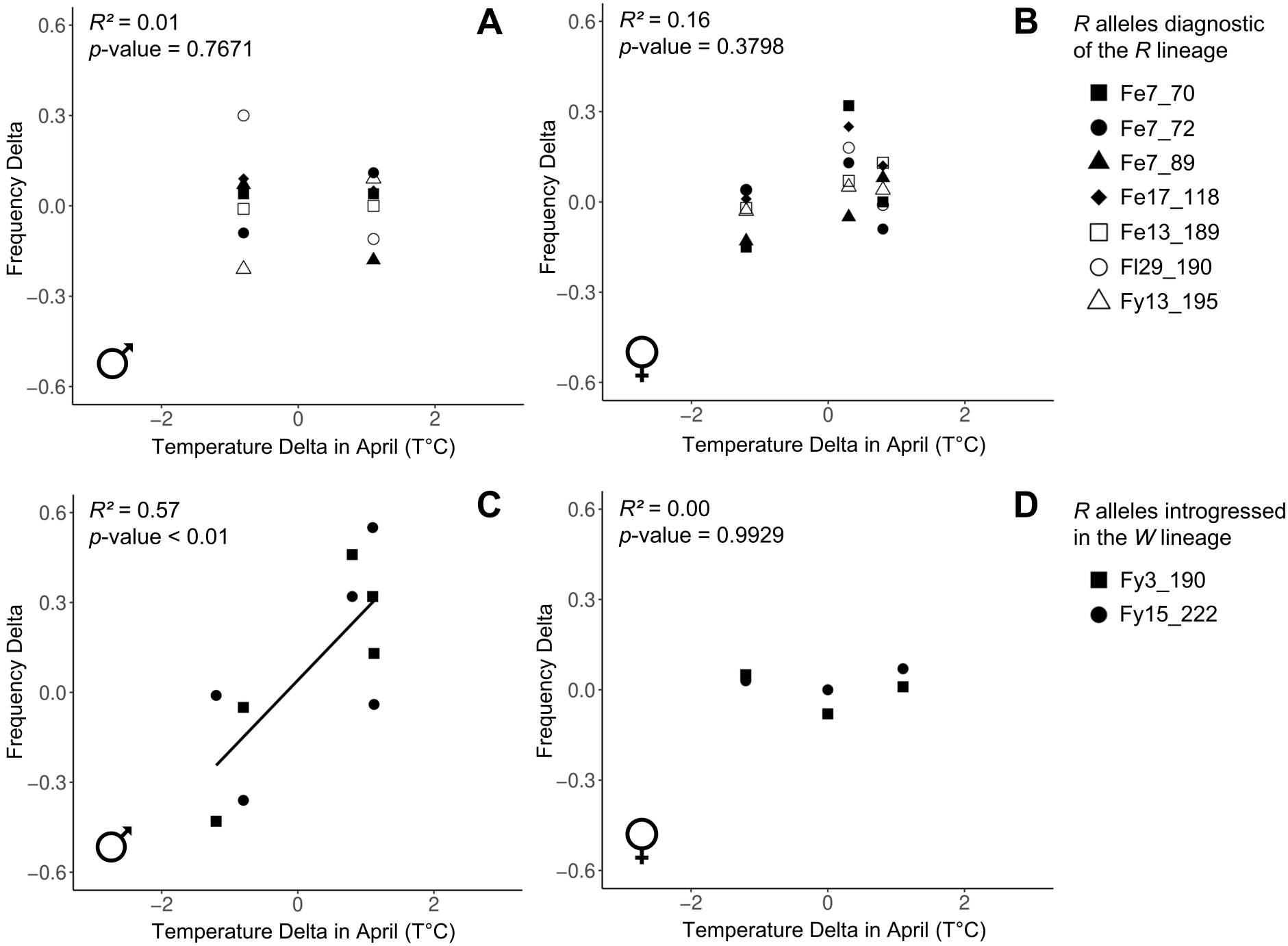
C


Cumulative hazard
200%
150%
100%
50%
0%
Pairwise log-rank test:
F. aquilonia vs F. polyctena < 0.001
F. aquilonia vs Hybrid < 0.001
F. polyctena vs Hybrid = 0.67
0
10
20
30
Time (min)

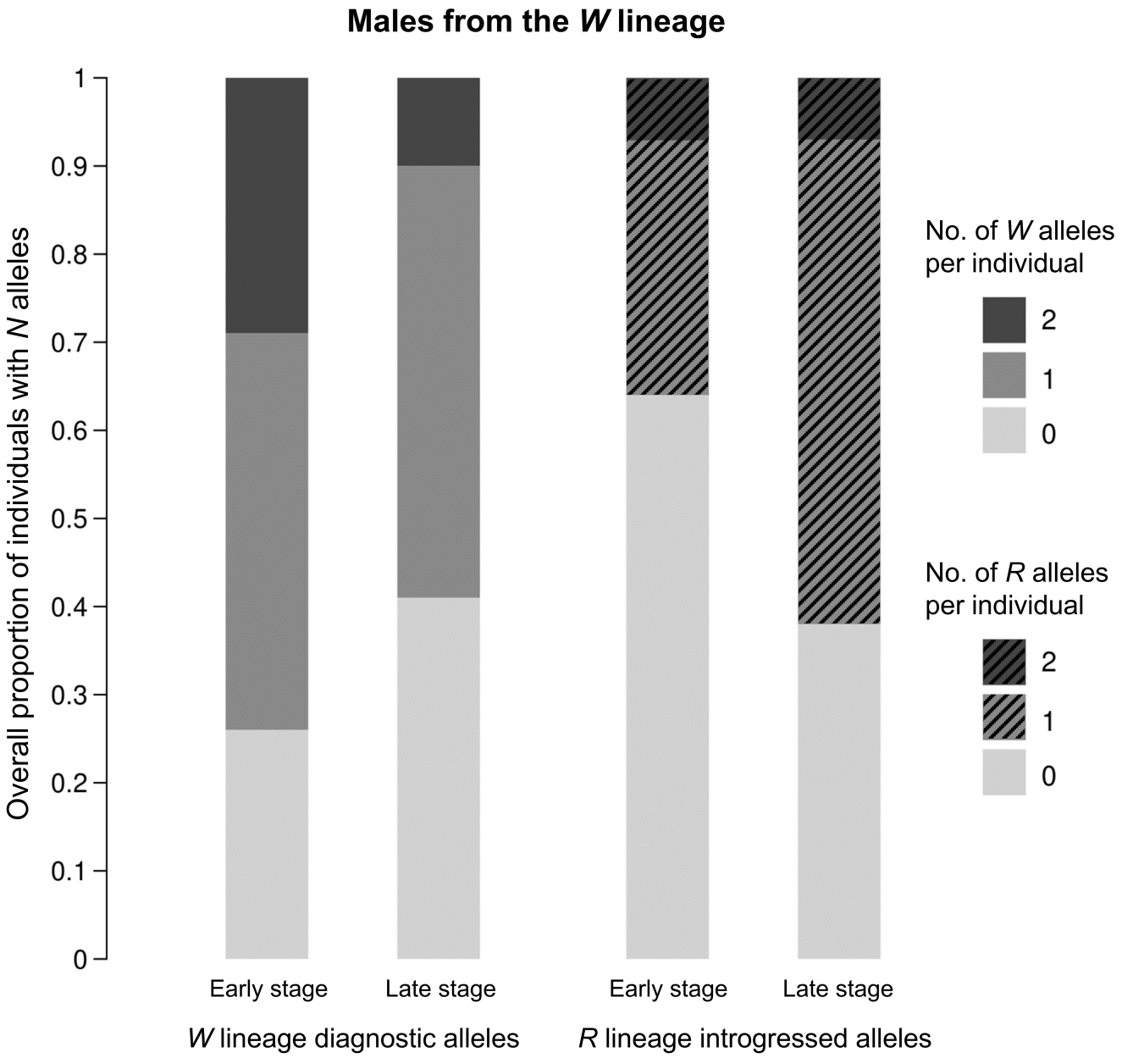
Figure


A
R^2 = 0.27
p-value = 0.1105
Frequency Delta
Temperature Delta in April (T°C)
B
R^2 = 0.01
p-value = 0.8865
Frequency Delta
Temperature Delta in April (T°C)
W alleles diagnostic of the W lineage
Fe7_66
Fe19_178
Fy3_188
Fy12_189
Fy13_205
Fy15_234
C
R^2 = 0.40
p-value < 0.05
Frequency Delta
Mean Minimum Temperature Delta in April (T°C)
D
R^2 = 0.10
p-value = 0.6303
Frequency Delta
Temperature Delta in April (T°C)
W alleles introgressed in the R lineage
Fe7_68
Fe17_110
Fl29_188
Fe13_198
Fy15_232

Figure


A
R^2 = 0.01
p-value = 0.7671
B
R^2 = 0.16
p-value = 0.3798
C
R^2 = 0.57
p-value < 0.01
D
R^2 = 0.00
p-value = 0.9929
Frequency Delta
Temperature Delta in April (T°C)
0.6
0.3
0.0
−0.3
−0.6
−2
0
2
♂
♀
R alleles diagnostic of the R lineage
Fe7_70
Fe7_72
Fe7_89
Fe17_118
Fe13_189
Fl29_190
Fy13_195
R alleles introgressed in the W lineage
Fy3_190
Fy15_222

Figure


Males from the W lineage
Overall proportion of individuals with N alleles
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Early stage
Late stage
Early stage
Late stage
W lineage diagnostic alleles
R lineage introgressed alleles
No. of W alleles per individual
2
1
0
No. of R alleles per individual
2
1
0

Differences in thermal tolerance between parental species could fuel thermal adaptation in hybrid wood ants

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Supplementary Material

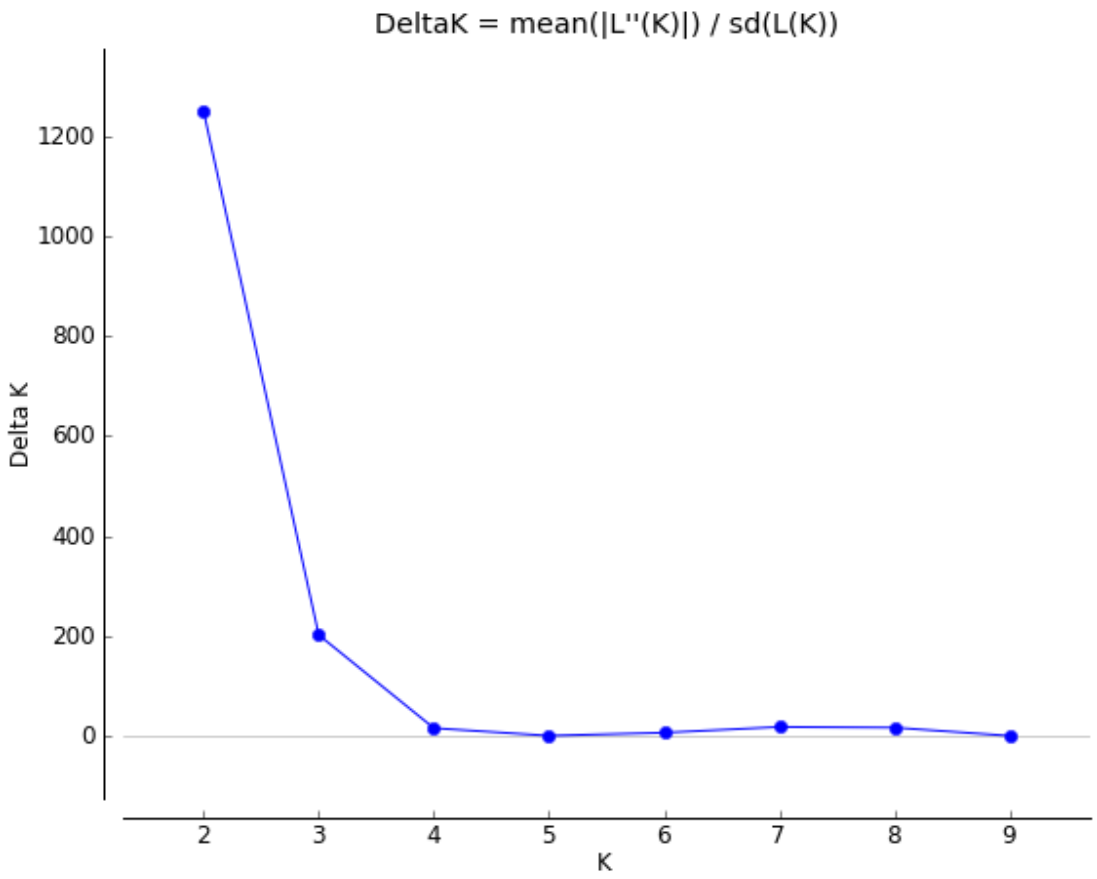

Figure S1: Results of the ΔK-statistics (y-axis) from STRUCTURE HARVESTER for samples used in thermal assays. STRUCTURE was run with the number of genetic clusters (K, x-axis) ranging from 1 to 10 and five iterations per run. K=2 has the highest ΔK value, suggesting this is the uppermost hierarchical level of structure in the individual-level microsatellite data.

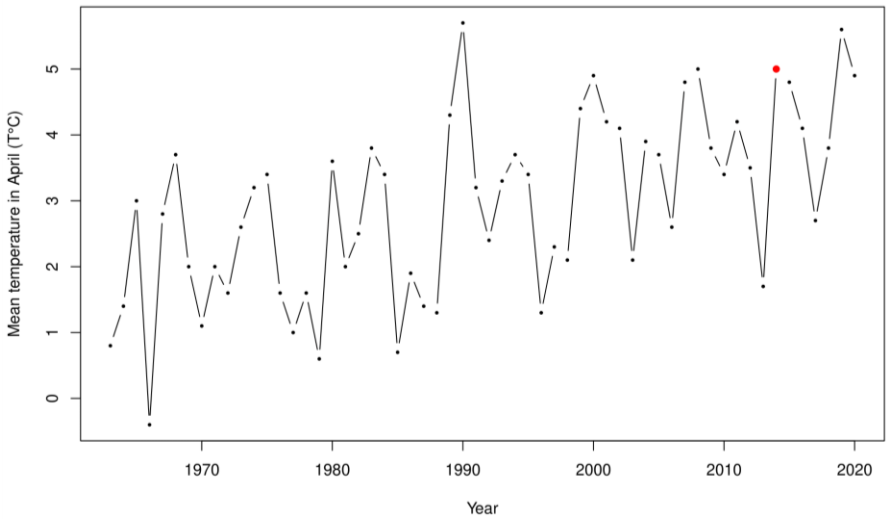

Figure S2: Mean April temperature at the Hanko Tvärminne station from 1963 to 2020, the 2014 year is represented in red (used to study allele frequency change from early to late developmental stage).

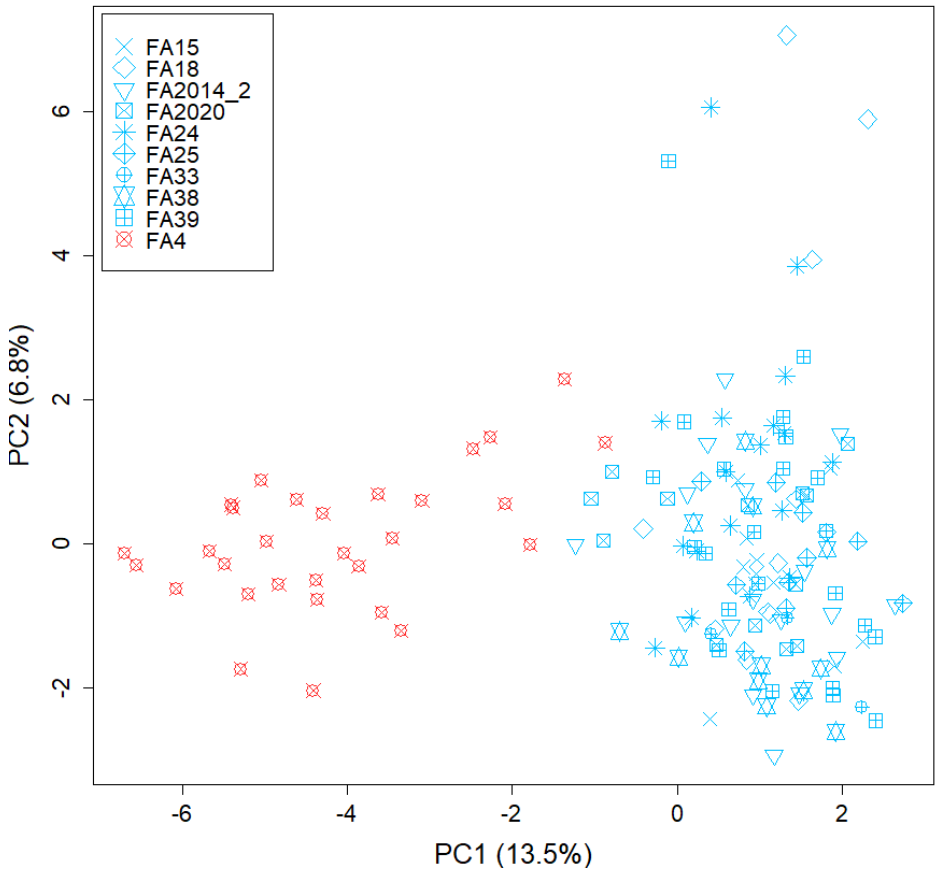

Figure S3: Principal component analysis of the Långholmen sampling location, in blue is represented the *W* lineage and in red the *R* lineage. Each nest is indicated by a symbol, each datapoint is an individual. Note that individuals from the *W* lineage come from the year 2018 and 2020. The two lineages are separated in two distinct groups and no substructure due to a nest effect or the year is present within the *W* lineage in blue.

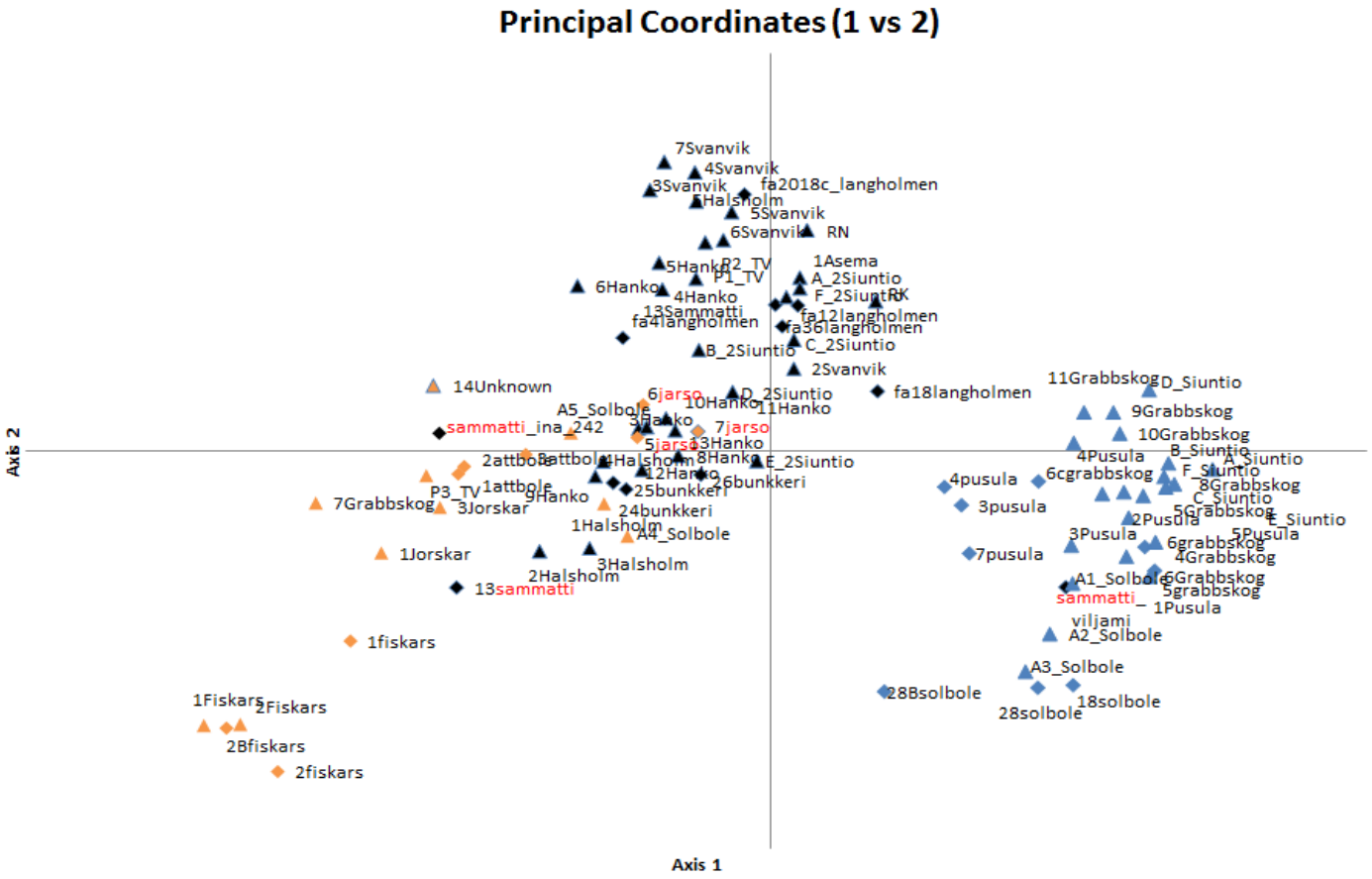

Figure S4: Principal coordinates analysis of nine microsatellite markers from 29 new nests (diamonds) and 66 previously sampled nests (triangles, Beresford et al. 2017) for the thermal assays. The data points are colored based on the preliminary information on species status (orange for *F. polyctena*, blue for *F. aquilonia* and black for hybrids). The first axis explains 30.8% of the variation and the second axis 10.3%. The first axis divides the samples according to parental gene pools, where Finnish *F. polyctena* individuals are situated to the left, Finnish *F. aquilonia* to the right and hybrids in the middle. Nests that were assigned to different groups than in Beresford et al. (2017, nests from Sammatti) are shown in red. Three nests from Jarsö that were initially classified as *F. polyctena* are also shown in red.

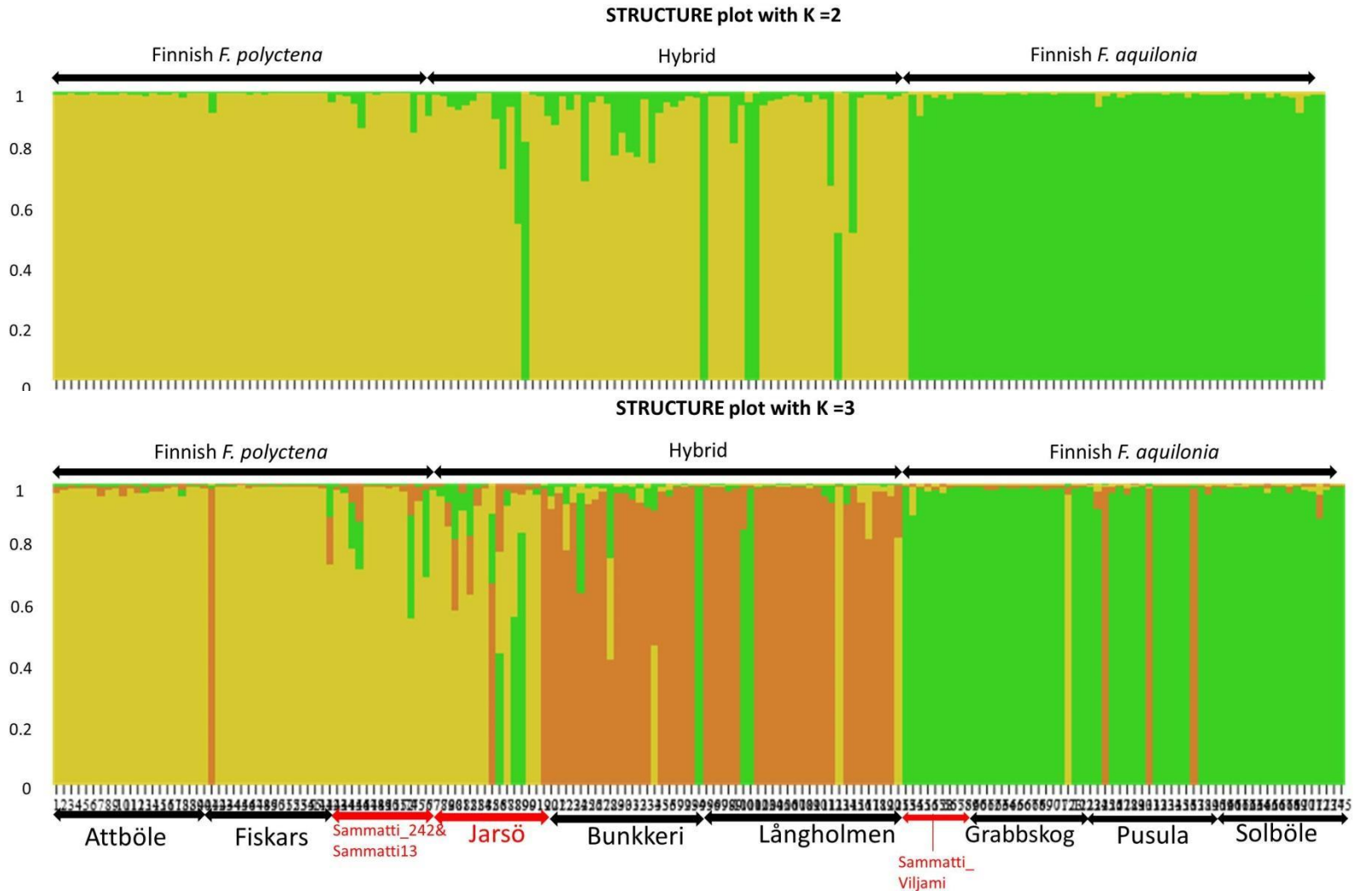

Figure S5: Results from the STRUCTURE assignment of 175 individuals used in thermal assays with K=2 and K=3. The three groups are identified into clusters with different levels of Finnish *F. aquilonia* (green) or Finnish *F. polyctena* (yellow) origin. In K=3, hybrids are identified with orange. Finnish *F. polyctena* populations are on the left, hybrids in the middle and Finnish *F. aquilonia* on the right. Different populations are visualized in the y-axis with black arrows. Y-axis indicates the percentage of assignment of each individual to the two clusters. Two nests from Sammatti (Sammatti_242 & Sammatti13) show signatures of Finnish *F. polyctena* origin, whereas the Sammatti_Viljami nest clusters with Finnish *F. aquilonia* individuals. The population from Jarsö shows a signature of mixed ancestry.

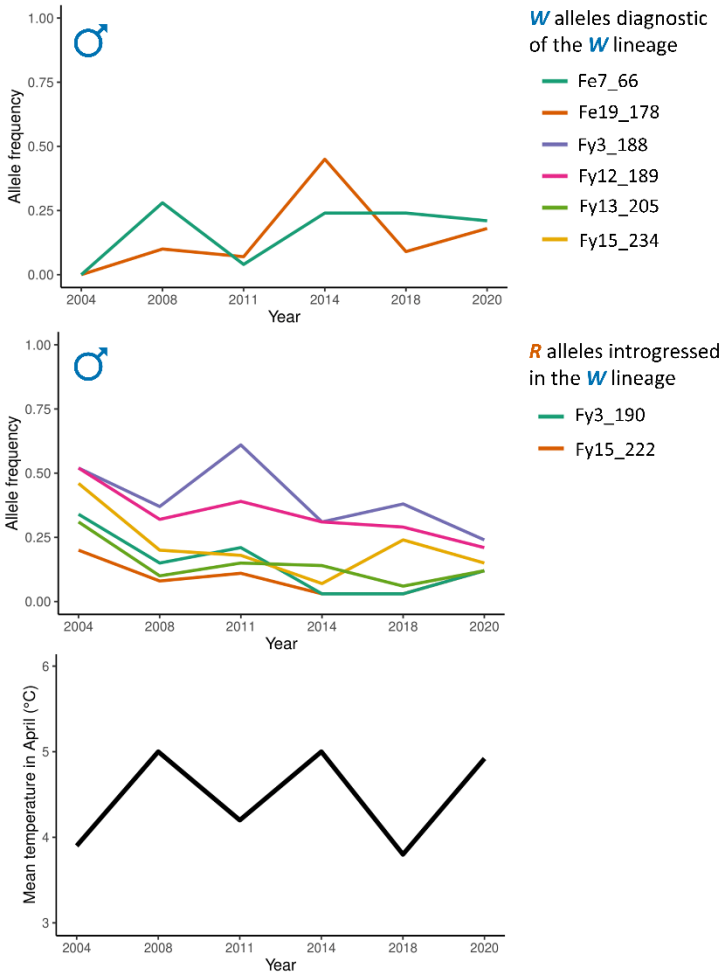

Figure S6: Allele frequency fluctuations of diagnostic and introgressed alleles in males from the *W* lineage and mean temperature during April across sampled years. With increasing temperature, we expected the *R* lineage alleles to increase and *W* lineage alleles to decrease, *vice versa* with decreasing temperature. These correlations were confirmed by the analysis performed in the main text.

Supplementary tables legends

Table S1: Final assignment of genotyped nests to Finnish *F. polyctena* (Pol), *F. aquilonia* (Aq) or hybrid (Hyb) of the samples used in thermal assays and number of individuals used in the thermal experiments. Identification was based on both PCoA and STRUCTURE analyses. Previously, nests were identified by either morphology or microsatellite and mitochondrial data (Beresford et al. 2017). Sampling schemes for the heat knock-down, chill-coma recovery and CTmax experiments are also indicated.

Table S2: Samples collected and genotyped from the Långholmen sample location over the years. The genetic groups of the Långholmen sampling location and mitochondrial haplotypes (from Kulmuni et al. 2010) are also indicated.

Table S3: Samples from the 2014 W lineage males at early and late development stage collected and genotyped from the Långholmen sample location and used in the differential survival of W males analysis.

Table S4: Raw allele frequencies at the 9 microsatellite markers used in the study for both males and females of the W lineage. Highlighted in blue are the diagnostic alleles of the W lineage and in orange are the introgressed R lineage alleles into the W lineage.

Table S5: Raw allele frequencies at the 9 microsatellite markers used in the study for both males and females of the R lineage. Highlighted in orange are the diagnostic alleles of the R lineage and in blue are the introgressed W lineage alleles into the R lineage.

Table S6: Linear Mixed Model (LMM) results for diagnostic and introgressed R and W lineage alleles in males and females. The LMM was done using the *lmer* function in the *lme4* package (Bates et al. 2015) assuming a Gaussian distribution (Shapiro-Wilk Tests: *p*-values > 0.05). *Temperature delta* and *locus* were used as fixed factors in the model, *year* was used as a random effect and the model was weighted using the *allele count*. *P*-values were calculated using *t* values assuming a normal distribution, the significance threshold being fixed at 5%.

Table S7: Regression table of the Cox proportional hazards model for the heat-knockdown resistance analysis. *F. polyctena* is used as a reference category in both models to analyze the group effect.

Table S8: Regression table of the Cox proportional hazards model for the chill-coma recovery analysis. *F. polyctena* is used as a reference category in both models to analyze the group effect.

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Supplementary_tables_Am_Nat_accepted.xlsx