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Evidence for climate-mediated range expansion of hybrid wood ants

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Abstract

Climate change challenges many species. To persist, species can shift ranges, respond plastically, and adapt. Yet, these responses require variation - often limited in natural populations. While hybridisation can quickly increase genetic variation, potentially facilitating climate adaptation, it is unclear how hybrids respond to climatic changes. Here, we combine whole-genome, life-history, and climatic data of 67 wood ant populations across Finland to assess whether *F. aquilonia* x *F. polyctena* hybrids have advantages in warming climates compared to their cold-adapted parent *F. aquilonia*. We found that hybrids are active and their offspring emerge earlier in spring, suggesting a competitive advantage over *F. aquilonia*. Hybrids also withstand acute temperatures relevant in nature better than *F. aquilonia*. Further, the hybrids' border has shifted 200 km northwards, coinciding with the expansion of climatic conditions suitable for hybrids. Our results provide compelling evidence that hybrids perform better under warming climates than their cold-adapted parent, and that recent climatic changes can lead to range expansion of hybrids.

Keywords

adaptation to climate change; *Formica rufa* complex; whole-genome sequencing; CT_{max} acute heat assay; hybridisation

Introduction

Climate change is ubiquitous and challenges nearly every species. Since 1900, average global temperatures have increased drastically (*1*), with 2023 and 2024 being the hottest years since records began (Copernicus CS3). Concurrently, the frequency of extreme events such as heat waves, droughts, and heavy rainfall has dramatically increased in the last decades (*1, 2*). The effects of higher temperatures and climate change are diverse (*3-5*) fuelling biodiversity loss (*6-8*). It is thus pressing and fundamental to understand and predict how species respond and persist under ongoing climate change.

Species can cope with changing climates through shifts in their habitat range, phenotypic plasticity, adaptation via genomic changes, or a combination thereof (*9*). Rapid range shifts are less likely for taxa with limited dispersal capacities, such as many plant and some animal species (*10, 11*). Phenotypic plasticity allows individuals to adjust their phenotype to different environments within an organism's lifetime and without an underlying genomic change (*12*). However, plasticity is finite, costly, and limited by the genomic makeup of an organism, and may constrain genomic adaptation (*13*) Hence, it is expected that many species will need to rely on adaptation to persist (*14*).

Adaptation requires time and standing or novel genomic variation. While genomic variation may be present in large populations or populations with high mutation rates, many natural populations are small – often due to anthropogenic activities – and, as a result, have low levels of genomic variation (*15*). Hybridisation – defined as interbreeding between different populations or species (*16*) – can provide a way to quickly increase genomic diversity, thereby increasing the adaptive potential of populations. For example, hybrid offspring of sunflowers *Helianthus annuus* and *H. petiolaris* can persist in extreme environments such as deserts or salt marshes while the parents cannot (*17*). Altogether, such elevated genomic and phenotypic variation in viable hybrids could make them better equipped to persist in rapidly changing environments (*18*).

To date, knowledge on the extent of hybridisation in natural populations is limited. Before access to whole genome data, the percentage of hybridisation was estimated to be approximately 10% in animals and 25% in plants (*19*). However, these estimates predate the advent of modern genomic analyses, and current rates are likely higher as hybrids frequently remain undetected (*19*). Whatever the current hybridisation rates are, they are expected to increase with natural and human-driven processes. Especially, a changing climate can cause range shifts (*14*) and increase the likelihood that previously isolated populations will interact (*20*). For example, a recent southward move in an Atlantic puffin population led to the formation of a hybrid population in the Arctic (*21*). Additionally, a changing climate can also lead to the movement of taxa as seen in hybrid chickadees moving northwards (*22*). These examples illustrate that hybrid populations are forming and moving with climate change. However, we lack understanding of whether hybridisation also promotes adaptation to climatic changes.

The *Formica* mound-building wood ants are one group where hybrids occur naturally and where hybridisation may shape the response to climate change. This relatively young group has diverged into up to 13 species (*23*) within the last 500,000 years (*24*). Five of these

occur in Finland with many of them hybridising naturally (see below and *25, 26*), and hybrid populations account for a significant proportion of wood ant populations in Finland (*27*).

Wood ants are keystone forest species. They are frequently found in temperate and boreal forests where they provide important ecosystem services such as re-cycling nutrients (*28*), protecting the forest against bark beetles (*29*), and supporting over 200 species (*28*). Notably, the species use similar habitats but differ in their social structures and dispersal abilities and also in their climatic adaptation. Three of the five species in Finland are mainly single-queened and conduct long-range dispersal (*Formica lugubris, F. pratensis, F. rufa*), while the other two are multiple-queened and conduct short-range dispersal (*F. aquilonia, F. polyctena*) (*25, 28*). However, the social structure and dispersal abilities do not reflect climatic adaptation: *Formica aquilonia* and *F. lugubris* are cold-adapted, while the other three are warm-adapted. All wood ants also form characteristic and semi-stationary mounds, which can remain in the same location for multiple years (*28*). They thus need to respond to changing climates to ensure their persistence.

In Southern Finland, *F. aquilonia* x *F. polyctena* hybrids (as of now “hybrids”) form stable populations. The parental species have diverged with gene flow mainly from warm-adapted *F. polyctena* into cold-adapted *F. aquilonia* suggesting that hybridisation has been ongoing (*30*). Also, some contemporary hybrid populations have formed in the early 1900s (*31*) and persisted ever since (*26*). These hybrid populations differ in some respects from their parental species. For example, hybrids are found in warmer and drier microclimates than populations of locally dominant *F. aquilonia* (*27*). Moreover, preliminary results suggest that hybrids withstand heat stress equally well as warm-adapted parent *F. polyctena* and significantly better than the cold-adapted parent *F. aquilonia (32)*. Also, hybrids harbour variation relevant for climate as selection favours warm-adapted alleles in warm spring and cold-adapted alleles in cold springs *(32)*. These findings suggest that hybrid wood ants may better cope with rapidly changing climate than parental species. However, extensive interdisciplinary analyses across space and time comparing hybrids and parents are needed to prove such broad-scale responses to a warming climate.

Here, we integrate whole-genome sequencing, life-history, acute heat stress, and climatic data on wood ant populations across Finland. This extensive and unique data set allows us to compare *F. aquilonia x F. polyctena* hybrid populations with co-occurring cold-adapted *F. aquilonia* populations. We show that hybrids have a greater genomic diversity, which can lead to adaptive benefits. Hybrids are also likely to outperform *F. aquilonia* due to an earlier onset of mound activity and emergence of sexuals in the spring and an increased tolerance to acute heat stress. Consistent with these advantages, hybrid populations have persisted over 18 years, become slightly more abundant, and expanded their range to the north. The expansion of hybrid range coincides with expansion of climatic conditions suitable for hybrids over the past 18 years. Overall, our data suggest that increasing temperatures due to climate change have facilitated a northward expansion of these hybrid wood ants. These findings highlight the major evolutionary and ecological role of hybrids, also in response to a rapidly changing climate.

Results and Discussion

Formica aquilonia x F. polyctena hybrids are among the most abundant wood ants in Finland

Our first goal was to determine the distribution of *F. aquilonia*, *F. polyctena*, and their hybrids across contemporary Finland. To do this, we sampled 92 wood ant populations in 2023 (‘current sampling’) and used whole genome sequencing to identify species and hybrids. We found 48 *Formica aquilonia* (52%) and 21 *Formica aquilonia x F. polyctena* hybrid (23%) populations, but no *F. polyctena* populations, contrasting earlier morphological studies which have also reported *F. polyctena* in Finland (*33, 34*). We also sampled the other species and additional hybrids with a frequency of <13% (Tab. S1-2, Fig. S1-2). We used whole-genome sequencing for species identification, and it remains a possibility that previous studies based on morphological identification have misidentified species.

We found hybrids more frequently than several wood ant species in Finland, and hybrids increased in frequency. In this data set, 21 populations were of *F. aquilonia* x *F. polyctena* hybrid origin, which also represents a 75% relative increase from the 12 populations observed in a previous study (‘previous sampling’) using a similar number of samples (N_{2023}=21/92 (23%); $N_{2005\text{-}2019}$=12/87 (12%); Tab. S2; (*27*)). This indicates that hybrids are persistent and one of the most abundant wood ant taxa in Finland. The ratio of hybrids compared to parental *F. aquilonia* or *F. polyctena* is thus frequent and of a similar or higher ratio compared with other hybridising species pairs (some European ant species: 12-31%, (*35*); *Heliconius* butterflies: 7-13%, (*36*)). For the remainder of this study, we focus on the comparison between *F. aquilonia* x *F. polyctena* hybrids and cold-adapted parent *F. aquilonia*.

Hybrids also have a higher genomic diversity than *F. aquilonia*. We calculated the observed heterozygosity using whole-genome data as a proxy for genomic diversity for samples collected across the sympatric area (i.e. area where hybrids and *F. aquilonia* co-occur; Welch’s *t*-test: H_O hybrid: 0.11; *F. aquilonia*: 0.08; t= -5.66, df = 23.15, p-value<0.001; Fig 1 A). These values are similar to the previous sampling (H_O hybrid: 0.10; *F. aquilonia*: 0.08; (*27*)). Higher genomic diversity can contribute to faster adaptation to climatic changes (*17, 18*).

We calculated the hybrid index and identified the mtDNA origin which showed that all *F. aquilonia x F. polyctena* populations are later-generation hybrids. The hybrid index ranged from 0.32 to 0.66 and indicates limited backcrossing (i.e., scale 0 to 1, where 0 means only *F. polyctena* alleles and 1 means only *F. aquilonia* alleles; 359 ancestry informative markers; Fig. S3 A; (*31, 37*)). In nine hybrid populations (43%), mtDNA originated from cold-adapted *F. aquilonia* mothers, and in 12 (57%) from warm-adapted *F. polyctena* mothers, thus being indistinguishable from 50:50. This result differs starkly from the ‘previous sampling’, which identified ten *F. polyctena* maternal lines (83%) but only two *F. aquilonia* maternal lines in hybrids (17%; (*27*)). We also calculated pairwise mtDNA differences between maternal lineages for hybrid samples originated from both species, which ranged from 76 to 86 mutations (Fig. S3 B).

Hybrids differ in life-history traits from the cold-adapted parent *F. aquilonia*

Long-term data on the activity of ants and the emergence of sexual offspring show that hybrids are active earlier in the season, and their offspring emerges earlier and in greater numbers than *F. aquilonia* offspring. This data was recorded in three hybrid populations and three *F. aquilonia* from March until June from 2020 to 2023. We found that hybrid mounds were active earlier in spring than *F. aquilonia* mounds (Fig. 1 B, relative proportions calculated over all years; proportion test; March, X^2 = 10.64, df = 1, p-value<0.001; April, X^2 = 11.56, df = 1, p-value<0.001; Mai, X^2 = 3.18, df = 1, p-value = 0.075; June, X^2 = 1.71, df = 1, p-value = 0.191; corrected for multiple testing). Also, offspring emerged from hybrid mounds in March, compared with *F. aquilonia* mounds where most individuals emerged in June (Fig. 1 C; relative percentages calculated for observed mounds; two-sample test for equality of proportions with continuity correction; March, X^2 <0.001, df = 1, p-value = 1.000; April, X^2 =8.32, df = 1, p-value = 0.004; May, X^2 = 19.87, df = 1, p-value<0.001; June X^2 = 22.54, df = 1, p-value<0.001).

Cross-sectional and long-term data show that the within-mound temperature differs between hybrids and *F. aquilonia*. The within-mound temperature from 69 populations was warmer in hybrid mounds than in *F. aquilonia* mounds (measured from the mound top; hybrids mean = 26.35 °C; *F. aquilonia* mean = 24.36 °C, Fig. 1 D; *t*-test = -2.133, df = 45.69, p-value = 0.038, after checking for a latitudinal effect). Similarly, the long-term within-mound temperature measured from 2020 to 2023 (using HOBO data loggers) from 6 populations was significantly higher in hybrid mounds than in *F. aquilonia* mounds for almost the entire year, except for June and November (Fig. 1 E; Tab. S3). In contrast, within-mound temperature was significantly warmer in *F. aquilonia* mounds in July and August than in hybrid mounds.

The difference of both taxa in the activity in spring is likely due to differences in the endogenous mechanisms, which mainly control diapause in wood ants (*38, 39*), as well as local adaption to climates (*38*). At the same time, temperature or photoperiod are expected to have little effect on diapause (*38, 39*). In Finland, *F. aquilonia* is likely adapted to long winters and having a longer diapause, while hybrids, with their genetic contribution of Southern European *F. polyctena*, seem to be adapted to shorter winters and having a shorter diapause. A shorter diapause and thus earlier activity allow hybrids to start foraging earlier in the season and enables the earlier emergence of sexual offspring. In turn, hybrids may have a competitive advantage in obtaining nest sites and food over *F. aquilonia* and also other wood ants in Finland.

The within-mound temperature in wood ants is produced by workers that produce warmth with their metabolic heat (*40*) and due to their associated microorganisms (*41*). During the season, workers also continuously regulate the nest temperature by their metabolism, partial removal of mound material to reduce wall thickness, and movement of queens and brood inside the mound (*42*). Hybrids' higher within-mound temperature, especially in the spring, could be an effect of differing behaviour and nest construction techniques. It could further be coupled with the endogenous control of the diapause and contribute to an earlier activity in spring contributing to a competitive advantage of hybrids over *F. aquilonia*.

Hybrids perform better under acute heat stress

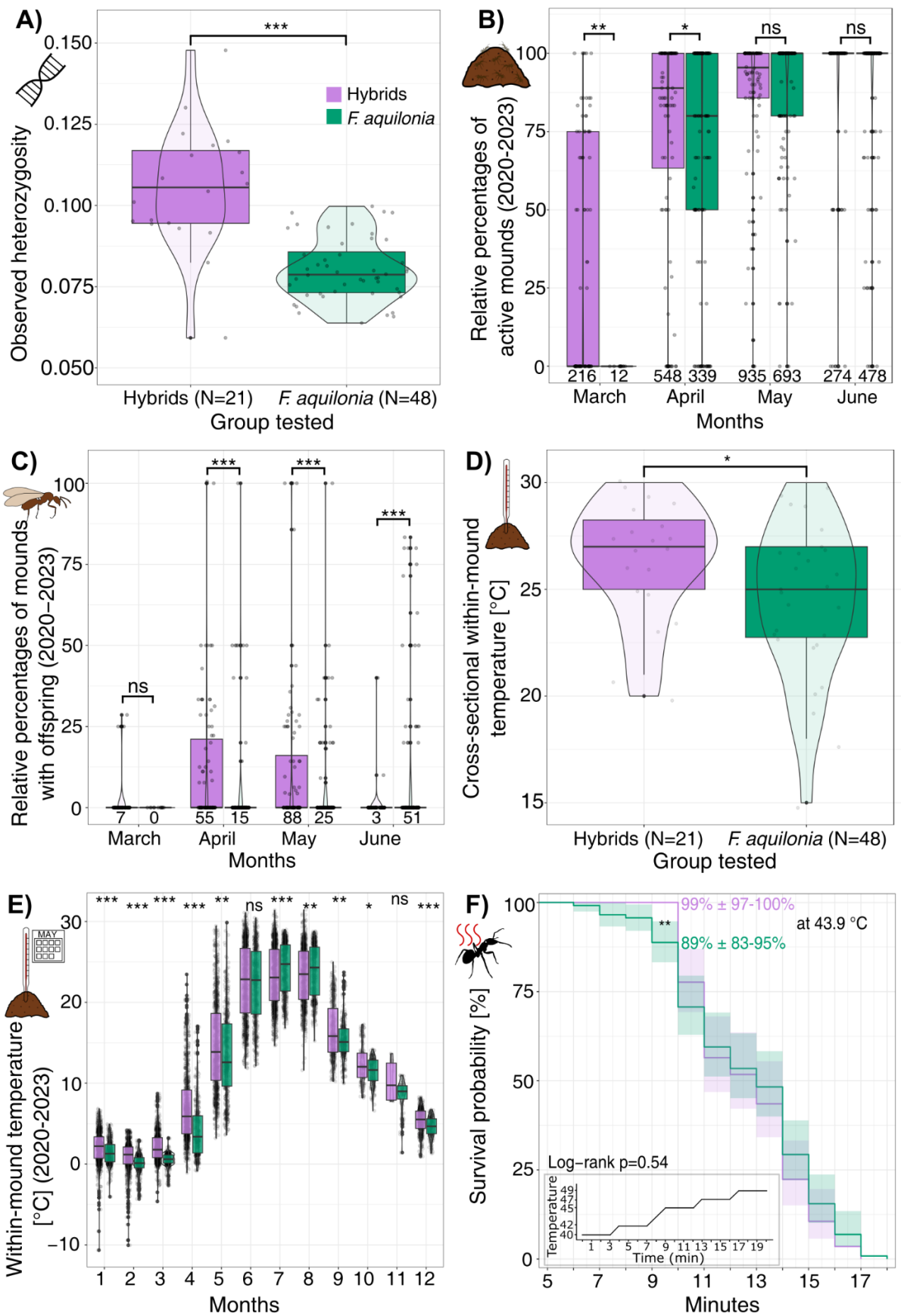
Given that the mounds of hybrids are warmer, we compared the performance of individual hybrid and *F. aquilonia* ants in experimental heat stress assays. We tested a total of 205 ants across 18 populations in a Critical Thermal Maximum assay (CT_{max}; (*43*)). In brief, we transferred ants to a 1.5 ml Eppendorf tube sealed with cotton and stored the tubes in a block heater (Eppendorf ThermoMix, Hamburg, Germany). We then increased the temperature from 40 to 49 °C in 20 minutes (Fig. 1 F inset) and recorded ants' status (active or knocked down) every minute.

The CT_{max} assay demonstrated that hybrids withstand acute heat significantly better than *F. aquilonia* at 43.9 °C in the short run (X^2 = 7.18, df = 1 p-value = 0.004; asterisk in Fig. 1 F, Tab. S5). This temperature is also close to wood ants' thermal maximum at ~45 °C. When assessing the survival probability over the entire 20 minutes of the assay, hybrids and cold-adapted *F. aquilonia* withstand acute heat stress equally well. This is regardless of whether the tested *F. aquilonia* workers originate from a sympatric area or from entire Finland (*sympatric area*, Kaplan-Meier survival analysis, p-value = 0.48, Fig. 1 F; *entire Finland* p-value = 0.57, Fig. S4 A).

We found no association of warm-adapted maternal mtDNA origin or the contribution of warm-adapted alleles in shaping acute heat tolerance of the hybrids. Hybrids with mtDNA of warm-adapted *F. polyctena* fell into heat coma at a similar temperature than hybrids with mtDNA of cold-adapted *F. aquilonia* (*F. polyctena*-mtDNA hybrids: 45.6 °C; *F. aquilonia*-mtDNA hybrids: 45.5 °C; one-sided Mann-Whitney-U-test, W = 856.0, p-value = 0.341; Fig. S4 B-C). Also, hybrid index as a proxy for higher genomic contribution from warm-adapted *F. polyctena* did not influence hybrids' performance under higher temperatures (linear regression, R^2 = 0.01, $F_{1, 87}$ = 1.901, p = 0.171, Fig. S4 D).

Our results demonstrate that hybrids perform better under acute heat stress than cold-adapted parent *F. aquilonia*. Previous results with a limited number of populations hinted that hybrids perform better under long-term heat exposure than *F. aquilonia (32)*, but knowledge on acute heat exposure for populations across latitudes was lacking. Here, we significantly increased the sample size (from 3 populations each to $N_{hybrids}$=21; $N_{F.\ aquilonia}$=29) as well as geographic area of where the workers originated (from 86 to 460 km maximum linear distance between the samples). Further, our approach better reflects naturally measured high temperatures in summer, where mound surface temperatures can reach over 70 °C.

One hypothesis for the hybrids' better performance under acute heat stress is that hybrids can more effectively express heat-shock proteins (HSPs). This has been observed in other warm-adapted ant species (*44*). Specifically, HSP60 and HSP90 are candidate HSPs, which are important for heat resistance in foraging ants in *F. cinerea* (*45*). Also, ants generally walk quicker and in straight lines on hot surfaces (*46*). Hybrids' higher "baseline" temperature and heat stress resistance may enable hybrid workers to walk longer on hot surfaces, even if only for a few seconds. In turn, this may allow hybrids to forage longer and more efficiently at hot surface temperatures than *F. aquilonia*. However, this hypothesis needs to be tested in future studies.


A)
Observed heterozygosity
0.150
0.125
0.100
0.075
0.050
***
Hybrids
F. aquilonia
Hybrids (N=21)
F. aquilonia (N=48)
Group tested
B)
Relative percentages of active mounds (2020-2023)
100
75
50
25
0
**
*
ns
ns
216 12
548 339
935 693
274 478
March
April
May
June
Months
C)
Relative percentages of mounds with offspring (2020–2023)
100
75
50
25
0
ns
***
***
***
7 0
55 15
88 25
3 51
March
April
May
June
Months
D)
Cross-sectional within-mound temperature [°C]
30
25
20
15
*
Hybrids (N=21)
F. aquilonia (N=48)
Group tested
E)
MAY
Within-mound temperature [°C] (2020-2023)
30
20
10
0
−10
*** *** *** *** ** ns *** ** ** * ns ***
1 2 3 4 5 6 7 8 9 10 11 12
Months
F)
Survival probability [%]
100
75
50
25
0
99% ± 97-100%
89% ± 83-95%
**
at 43.9 °C
Log−rank p=0.54
Temperature
49
47
45
42
40
1 3 5 7 9 11 13 15 17 19
Time (min)
5 7 9 11 13 15 17
Minutes

264

Fig. 1. Recorded traits and characteristics for F. aquilonia x F. polyctena hybrids (purple) and F. aquilonia (green) populations. A) Observed heterozygosity including a mean comparison between hybrids and *F. aquilonia*. B) Relative percentages of active mounds in Southern Finland recorded between March and June for the years 2020 to 2023 for hybrids and *F. aquilonia*. C) Relative percentages of mounds with offspring in Southern Finland observed from 2020 to 2023. The numbers below the boxplots in panel B) represent the numbers of mounds checked for B) and C). The numbers below the boxplots in panel B) represent the numbers of mounds with offspring observed. D) Within-mound temperature measured at the top of the mound recorded during the sampling in the field in the year 2023. E) Within-mound temperature measured from HOBO loggers between 2020-2023 between hybrids and *F. aquilonia* (HOBO data points for hybrid mounds for each month over the three years = 837, 756, 837, 870, 961, 930, 961, 793, 383, 124, 8, 372; HOBO data points for *F. aquilonia* mounds each month over the three years = 341, 336, 372, 360, 480, 480, 496, 440, 293, 176, 76, 186). F) Survival probabilities from the survival analysis for hybrids and *F. aquilonia* (workers tested, N hybrids = 89; N *F. aquilonia* = 116). The asterisks represent differences between the survival probabilities at minute 9 (43.9 °C; test of given proportions) with respective survival probabilities for hybrids and *F. aquilonia*. The inset in F) displays the CT_{max} temperature increase over 20 minutes.

Hybrids have persisted and expanded 200 km northwards

To assess species and hybrid persistence over a total of 18 years, we compared this 'current' data sampled in 2023 to 44 populations from the 'previous' sampling that were already identified in Satokangas et al. (2023; (*27*)) and mainly sampled in 2005-2008. We found the same species or hybrids in 32 populations (73%) and species' turnover in the other 12 populations (27%; Tab. S4). These revisited sites included ten hybrid populations, and we found that hybrids persisted in seven (70%) populations, some of these since 2005. Previous studies have indicated two isolated hybrid populations in Southern Finland have persisted for at least 19 years (*26*). These hybrid populations are estimated to have formed independently 13 to 47 generations ago, roughly at the beginning of 1900 (*31*). Here, we show, for the first time, that also other hybrid populations in Southern and Central Finland have persisted over time.

We found that the hybrid border has expanded northwards by ~200 km in a timeframe of 18 years (Fig. 2 A). The previous sampling was performed mainly in 2005-2008 with some additional populations sampled until 2019 (*27*). In the current sampling, it was 1.6 times more likely to find hybrid populations at higher latitudes than cold-adapted species, compared to the previous sampling (multinomial logistic regression; AICc = 238.30, Nagelkerke Pseudo-R^2 = 0.38, odds ratio z-value = -4.39, p-value <0.001; delta AICc intercept-only model: 35.58; Fig. S4 E). In contrast, we found no evidence that the range of either parental species or other warm-adapted species has changed (*F. aquilonia* no change; *F. pratensis* ~145 m northwards shift, *F. rufa* ~200 km southward shift; odds ratio z-value = -0.28, p-value = 0.078; Fig. 2 A). This indicates that the significant northwards shift is specific to hybrids.

The northward expansion of the hybrids coincides with expansion of climatic conditions suitable for hybrids

If climatic changes have allowed a northward expansion of hybrids, we expect that the climate in the 'expanded' range has changed in a way that is favourable for hybrids. Specifically, we expect that the expanded range, where hybrids have been found in 2023, should have a similar climate compared to that of the 'hybrid' range in 2005. In contrast, the

climate in the ‘expanded’ range in 2005, where hybrids were absent, should be unsuitable and different from the climate in the hybrid range in 2005 (Fig. 2 B). To test these predictions, we compared 5-year averages of six climatic variables (i.e., winter mean temperature, spring mean temperature, winter mean precipitation, spring mean precipitation, winter number of days above 0 °C and spring number of days below 0 °C. Note, we use the terms ‘2023’ and ‘2005’ for simplicity when referring to the five-year averages from 2018-2022 and 2000-2004). Here, we only used hybrid populations, assuming that hybrid populations could follow these environmental changes and that the climates are suitable for hybrids. We calculated 5-year averages to account for yearly environmental fluctuations.

Following our expectations, the winter in 2023 in the ‘expanded’ range (with hybrids present) was similarly warm and had a similar number of days above 0 °C as the winter in 2005 in the ‘hybrid’ range at lower latitudes (Fig. 2 C-D; Tab. S6). At the same time, and also in line with our expectations, winter in 2005 in the ‘expanded’ range (with hybrids absent) was colder and not suitable for hybrids. However, not all aspects of the climate in the ‘expanded’ range have changed in such a way that matches the hybrid niche. Specifically, in the spring in 2023, temperatures in the ‘expanded range’ were still lower, there were more frost days below 0 °C, and there was more precipitation than in the spring in 2005 in the ‘hybrid’ (Fig. S5 A-D; Tab. S6).

We also assessed whether the climate has overall changed in hybrid and *F. aquilonia* populations. For this, we compared the same six environmental variables described above within and between hybrid and *F. aquilonia* populations and between previous and current sampling. Briefly, the climate has changed similarly in both hybrid and *F. aquilonia* populations and followed the predicted climatic changes, with warmer and wetter winters and springs. Notably, these climatic changes were more pronounced in hybrid populations than in *F. aquilonia* populations (Fig. S6 A-E, Tab. S7). This suggests both hybrids and *F. aquilonia* will need to cope with rapid changes as climate warming continues.

From these results, we can conclude that the climate appears to have become more favourable for hybrids in northern areas, but not in all respects. The increased winter temperatures follow our expectation and indicate that warmer winters may be more important for hybrids to expand northwards and persist than cold spells in spring, and that cold spells do not limit their persistence at higher latitudes. This is also supported by a study on *Aphaenogaster* ants demonstrating that increasing minimum temperatures along an elevation gradient in North America allowed the ant *A. rudis* to move upward in elevation (*47*). Besides climate change, mild winter owed to the Baltic Sea may also contribute to the persistence of hybrids. Such mild winters had, for example, also a positive effect on the number of bird species in Southern Finland in the last decade (*48*).

Smaller mound volume and less divergent mtDNA at higher latitudes indicate a recent northward expansion of hybrid populations

If hybrid populations have initially formed in Southern Finland and have recently expanded northwards or if the northward expansion is due to new hybridisation events, we expect hybrid mounds at higher latitudes to be smaller. We also expect hybrid mtDNA in the expanded range to be less diverse than at lower latitudes since recently expanded hybrids

had less time to accumulate mutations. First, we found that hybrid mounds are smaller at higher latitudes (hybrids: 9085.7 South and 5235.4 North; Welch's t-test, t = 1.96, df = 17.33, p-value = 0.033; linear regression: R^2 = 0.02, estimate = -1009.40, F-statistic = 1.32, df = 1 and 18, t-value = -1.15, p-value = 0.265; Fig. 2 E), while *F. aquilonia* mounds are bigger at higher latitudes (*F. aquilonia* means: 8191.9 South and 12112.3 North; Mann-Whitney-U-test, W = 196, p-value = 0.057; linear regression: R^2 = 0.08, estimate = 875.10, F-statistic = 4.77, df = 1 and 45, t-value = 2.19, p-value = 0.034). This implies that hybrids have started building mounds at higher latitudes more recently.

Second, we found that mtDNA of hybrids is less diverse at higher latitudes than at lower latitudes (i.e., fewer pairwise mutations in the mtDNA within the taxa; linear regression, hybrids: R^2 = 0.03, estimate = -0.9825, F-statistic = 17.91, df = 1 and 570, t-value = -4.23, p-value<0.001). In *F. aquilonia*, we found the opposite trend with a higher divergence at higher latitudes (linear regression; *F. aquilonia*: R^2 = 0.03, estimate = 0.380, F-statistic = 211.70, df = 1 and 7308, t-value = 14.55, p-value <0.001; Fig. 2 F). This may be a signal of lower population densities in Southern Finland, which marks the Southern range edge of *F. aquilonia.*

Previous work hinted that selection favours alleles from the cold-adapted parent in cold springs and alleles from the warm-adapted parent in warm springs *(32)*. If such a signal is strong, hybrids may have a higher allele frequency of cold-adapted *F. aquilonia* alleles or *F. aquilonia* mtDNA origin at higher latitudes. However, the hybrid index and mtDNA origin were not correlated with latitude (hybrid index linear regression, R^2 = -0.02, estimate = -0.01, F-statistic = 0.36, df = 1 and 40, t-value = -0.50, p-value = 0.622, Fig. S7 A; mtDNA origin ANOVA, F-value = 0.979, df = 1 and 18, p-value = 0.336, Fig. S7 B). Nevertheless, we acknowledge that certain genomic regions and/or loci involved in the northward expansion and adaptation to cooler regions may not have been detected by simply looking at the ancestry proportion of the hybrid genomes.

These findings above support the idea that hybrids have recently expanded northward. Hybrids and both parental species have similar colony structures and probably also similar dispersal strategies. They are poor at long-range dispersal via flights (*49*) and new mounds are usually established by budding (i.e., queens and workers walk a few meters and found a new mound; (*50*)). Budding thus allows a slow but continuous northward expansion but does not explain the recent 200 km expansion.

In both parental species, males also disperse by flight (*51*), which can explain the northward expansion in hybrids – assuming that this dispersal trait is inherited. If this were true, we would expect to find backcrosses at higher latitudes, which we did not observe, making this scenario less plausible. Alternatively, hybrid populations may form via novel hybridisation events, but since *F. polyctena* is mainly absent from Finland, this also seem unlikely. It may thus be that hybrids differ in their dispersal strategy from their parental species, and that both hybrids' queens and males have the ability to disperse by flight. Alternatively, human-assisted dispersal of queens, which has been shown in a moving hybrid zone in *Solenopsis* ants (*52*) may lead to the establishment of hybrid populations at higher latitudes. These scenarios need to be addressed in future studies.

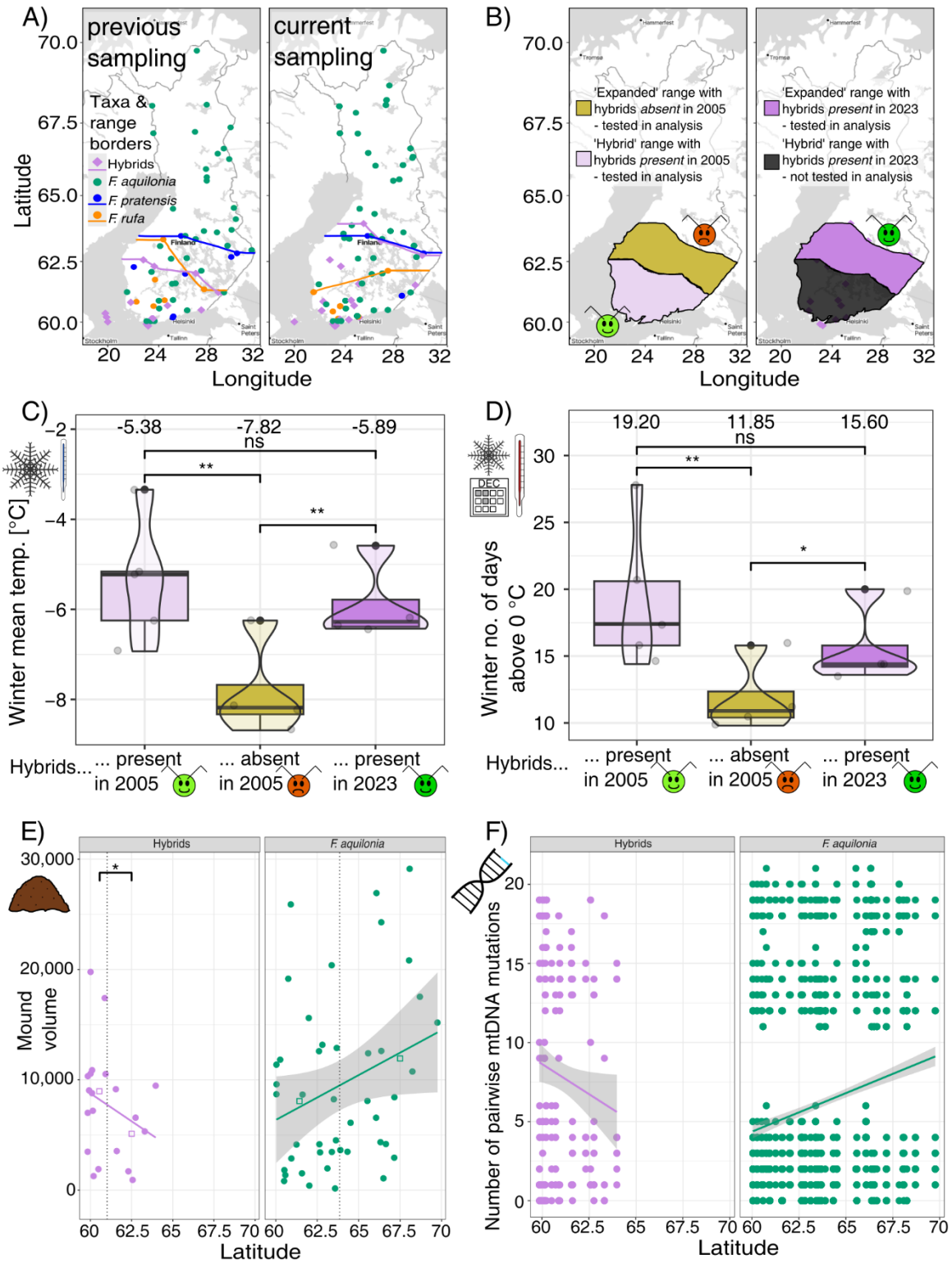


Fig 2. Sampling map, comparison of 5-year climatic averages of hybrid areas, and mound volume and pairwise mutations plotted against latitude. A) Current and previous sampling map including taxa assignment based on whole-genome sequence data to identify wood ant species and hybrids. The previous sampling map was modified from Satokangas et al., (2023; (27)). The current sampling originates from this data set. The lines represent species and hybrid borders. B) Maps displaying the 'hybrid' range and 'expanded' range where hybrids were found and which were used for the temporal comparison. In C) and D), 5-year average of climatic variables were calculated backward from the sampling year 2005 (period 1999-2004) and 2023 (period 2018-2022). For

simplicity, we refer to them as 2005 and 2023. C) Winter mean temperature and D) Number of days in winter above 0 °C (warm winter days). 'Winter' is defined as the diapause period between December to February. Values above the boxplots represent mean values. Asterisks represent bootstrapped p-values between hybrid areas at higher and lower latitudes and over time (Tab. S6) with * <0.05, ** <0.01, and ***<0.001. N suitable in 2005 = 5. N unsuitable in 2005 = 4. N suitable in 2023 = 4. E) Calculated mound volume (based on mound length, width, and height) plotted against latitude for hybrids (N=21) and *F. aquilonia* (N=48). The dotted lines represent the latitudinal means for both hybrids and *F. aquilonia*. They split the data in a "northern" and "southern" area used to compare the means, which are represented as squares. F) Number of pairwise mutations within hybrids and *F. aquilonia* separately plotted against the latitude for hybrids (N=572) and *F. aquilonia* (N=7310). In F), we combined data from the 'current' and 'previous' sampling to increase statistical power.

Conclusions and broader implications

Here we combine whole-genome data of wood ants sampled in 2023 across Finland, with life-history, cross-sectional and long-term environmental, acute heat data, as well as climatic data and demonstrate that (1) *F. aquilonia x F. polyctena* hybrids have a higher genomic diversity than its cold-adapted parent *F. aquilonia*, hybrids are active with sexual offspring emerging earlier in the spring than in *F. aquilonia*. Hybrids also have an increased tolerance to heat stress; (2) hybrids have persisted and expanded approximately 200 km northwards in the last 18 years; and (3) this northward expansion of the hybrids coincides with extension of winter conditions suitable for hybrids. The persistence and northward expansion of hybrids at higher latitudes is probably the result of both climate change and the hybrids' genomic composition and their corresponding shift in life-history traits. Future studies should address if hybridisation and persistence of hybrids in this ant group is as frequent outside of Finland as discovered here.

With ongoing climate change (*1*), hybridisation is expected to increase in frequency (*14*) due to an increase in secondary contact of species and/or populations. Here, we show that hybrid wood ants with limited dispersal abilities can have competitive advantages over resident species in the face of changing climates (*17*). Hybrids are also able to track rapid changes in climate, which allows them to expand their distribution. They can thus be well-equipped to respond to or withstand climatic changes – possibly better than their parental species – as seen with these hybrid ants and in other hybrid organisms such as in oaks (*53*), birds (*22*), and fish (*54*). Further, wood ants are keystone species of boreal forests, supporting a wealth of biodiversity (*28*). Coupled with their abundance, wood ant hybrids play a major evolutionary and ecological role in the forest ecosystem. We argue that hybrids should also be considered in conservation planning (*55*), especially if they have a higher adaptive potential than parental species.

442 **Materials and Methods**

443 Sampling of workers and recording of environmental data

444 From June to August 2023, *Formica* wood ant mounds were sampled from 92 populations
445 across Finland (Fig. 2A, Tab. S7; map created using ggplot2 (*56*), ggpubr (*57*), and ggsignif
446 (*58*) in R v4.3.0 and RStudio v2025.09.0+387). From each mound, approx. ten workers were
447 collected alive for thermal tolerance assays (detailed below), and a handful of workers were
448 collected and stored in 96% ethanol for genomic analyses. Whenever possible, workers from
449 the same mound and species were collected in 2023 as used in Satokangas et al. (2023;
450 (*27*)) for genomic and ecological analyses. During sampling, we recorded the within-mound
451 temperature and mound size for each mound. We measured within-mound temperature from
452 the highest point of the mound using a core thermometer (Xavax 111381, Monheim,
453 Germany; Tab. S1). We also measured the mound length, width, and height using a
454 measuring tape and calculated the mound volume following (*59*) and using the equation: 2/3
455 × π × D/2 × d/2 × H where D is the length, d is the width, and H the height of the mound.

456

457 Bioinformatic analyses

458 *DNA extraction, whole-genome sequencing, and variant identification*

459 To identify hybrids and different species, we extracted DNA from a single diploid worker per
460 mound and population (N=98) using the Qiagen DNEasy Blood and Tissue kit (Qiagen,
461 Hilden, Germany; N=81) following the manufacturer's protocol or a modified CTAB extraction
462 protocol (N=17; (*60*)). In both procedures, DNA was eluted in 100 µl AE elution buffer from
463 the Qiagen kit. Library preparation and whole-genome sequencing were outsourced to a
464 commercial provider (Novogene, UK). Whole-genome sequencing was conducted on an
465 Illumina NovaSeq X Plus with 150bp paired-end sequencing.

466 Raw Illumina reads and adapter sequences were trimmed using trimmomatic (v0.39; (*61*))
467 before mapping against a Finnish *F. aquilonia* × *F. polyctena* hybrid reference genome (*62*)
468 using bwa mem (v0.7.17; (*63*)). Using picard tools (v2.27.5;
469 http://broadinstitute.github.io/picard), duplicates were removed with the command
470 "MarkDuplicates", read groups were added with the command "AddOrReplaceReadGroups",
471 and bam files were indexed using "samtools index" in samtools v1.2.1 (*64*). The average
472 read depth was 15.7×, as computed across individuals using mosdepth (v0.3.6; (*65*)) after
473 duplicate removal. Overlaps were clipped using "bamUtil" and the function "bam clipOverlap"
474 (v1.0.15, https://github.com/statgen/bamUtil/releases).

475

476 *SNP calling and filtering data*

477 For single nucleotide polymorphism (SNPs) calling, we used these 98 samples ('current
478 sampling') plus additional 85 samples from a 'previous sampling' from Satokangas et al.
479 (2023) (*27*) as a reference. We called SNPs with the freebayes software v1.3.6 (*66*). To
480 speed up the SNP calling, 50 kb regions of the reference genome were defined using the

481 script “split_ref_by_bai_datasize.py” (available under
482 https://github.com/freebayes/freebayes/blob/master/scripts/split_ref_by_bai_datasize.py;
483 main settings: --k option to disable population priors; --genotype-qualities to write genotype
484 qualities to a file; --skip-coverage to skip high coverage regions; --limit-coverage 100 to only
485 use regions with a maximum coverage of 100; --use-best-n-alleles 3 to only consider 3
486 alleles to reduce runtime). The resulting VCF file was normalised using bcftools norm-
487 function (v1.10; (*67*)). Using bcftools, sites located at less than two base pairs from InDels
488 were excluded, as well as sites that were supported by only Forward or Reverse reads.
489 Furthermore, we only kept biallelic SNPs with quality values equal to or higher than 30 and,
490 we filtered sites based on individual sequencing depth: Genotypes were marked as missing
491 when their depth exceeded twice the mean depth of the individual in question. Genotyping
492 errors (e.g., misaligned reads) were removed by excluding sites that displayed heterozygote
493 excess after pooling all samples using vcftools v0.1.17 (*68*). Individual genotypes with a
494 quality of lower than or equal to 30 and a depth of coverage lower than or equal 8 were set
495 as missing data. Sites with more than 10% of missing data over all samples were removed
496 from the next steps. Lastly, singletons were removed (minor allele count <2) using vcftools.

497 After these filtering steps, 3,324,311 SNPs were kept from 179 individuals with sequencing
498 depths of at least 8×, including 92 out of the 98 samples from the ‘current sampling’ in 2023.
499 Using vcftools, the final dataset was thinned with 20 kb distances to minimise linkage
500 disequilibrium. We further excluded chromosome three (the social chromosome with
501 reduced recombination due to supergene inversions; (*69*)) as well as chromosome ‘zero’,
502 which contains all unscaffolded contigs with no known genomic location (*62*). The final data
503 set resulted in 9,979 genome-wide SNPs used for the genomic analyses described below.

504

505 Species and hybrid identification and downstream genomic analyses

506 *Principal component analysis (PCA), phylogenetic network and admixture analyses*

507 To assess the species’ identity, genomic structure and hybrid status, a principal component
508 analysis (PCA) was conducted in plink (v2.00a6.12, (*70*)) using the nuclear genomic SNPs
509 data from all samples. We expected that genomically diverged species and hybrids would
510 form separate clusters in the PCA.

511 To corroborate species identity and hybrid status, we further constructed a phylogenetic
512 network using the genomic SNP data from all samples. In such a phylogenetic network,
513 genomically similar samples cluster closer to each other regardless of geographic origin. For
514 the network, pairwise genomic distance between all samples was calculated using the script
515 distMat.py from github.com/simonhmartin/genomics_general. Using SplitsTree (v. 6.3.37;
516 (*71*)), we generated a phylogenetic network using the NeighbourNet approach (*72*).

517 To assess the extent of admixture and thus hybridisation in the samples, we performed an
518 admixture analysis (*73*) with the 9,979 nuclear SNP data. For admixture, the number of
519 clusters K was chosen to range from K = 2 to 10, without prior knowledge of taxat but
520 including samples and species identity from Satokangas et al. (2023) (*27*). The most likely
521 value of K was chosen based on the lowest cross-validation error rates. We further extracted
522 the allele frequencies from the most likely K to be used in statistical analyses.

523

524 *Mitochondrial haplotype network*

525 To create a haplotype network using the mitochondrial DNA only, we called SNPs from the
526 mitochondrial genome using all 179 samples. We used the same filtering steps as with the
527 nuclear DNA. After filtering, 466 SNPs were kept. A consensus sequence for each sample
528 across all samples was created by converting the VCF file into a fasta-file using the faidx
529 function in samtools. Next, the fasta-file was converted to a phylip-format and used to create
530 a median-joining network in PopArt (*74*). Species that share identical or similar mitochondrial
531 haplotypes should cluster more closely together in the network.

532

533 *Selecting hybrids and F. aquilonia for subsequent comparisons*

534 After reliably identifying species and hybrids, we only used samples from *F. aquilonia x F.*
535 *polyctena* hybrids and its cold-adapted *F. aquilonia* parent for subsequent analyses. We
536 excluded the warm-adapted parent *F. polyctena* because it was not found in the data set.
537 We also excluded the warm-adapted *F. rufa* and *F. pratensis*, as well as cold-adapted *F.*
538 *lugubris* because they are either single-queened (or facultatively have only a few queens)
539 and not as dominant locally as *F. aquilonia* or hybrid. Both *F. aquilonia* and hybrids are also
540 socially more similar (i.e., both are multiple-queened and form supercolonies; (*75*)).

541

542 *Observed heterozygosity, hybrid index and intraclass heterzoygosity calculation*

543 To assess whether hybrids had a greater genomic diversity than *F. aquilonia*, we first
544 calculated the observed heterozygosity for each sample from the current sampling using
545 vcftools and then tested if their means differed significantly using a Welch's t-test. To
546 calculate the hybrid index for the hybrid samples, we used the previous and current sampling
547 and filtered the VCF-file to only keep the parental species *F. aquilonia* and *F. polyctena* and
548 the *F. aquilonia* x *F. polyctena* hybrids. Then, we removed monomorphic sites, selected
549 ancestry informative markers (AIMs) with an allele frequency difference of 0.95 between *F.*
550 *aquilonia* and *F. polyctena* (*62*), and filtered the data to keep only markers with less than 5%
551 missing data. Lastly, we thinned the VCF file to 1 SNP/500 kb yielding 359 SNPs. We then
552 exported the VCF file and calculated the hybrid index for each individual as following: sum of
553 *F. aquilonia* alleles / ((sum of homozygous *F. aquilonia* and homozygous *F. polyctena*
554 alleles) - 2 * number of missing genotypes). Using the hybrid index and individuals from the
555 current sampling 2023, we conducted a linear regression testing whether the hybrid index is
556 associated with the latitude as well as a linear regression testing whether it is associated
557 with the temperature when ants fall into heat coma (section "*Critical Thermal Maximum*
558 *(CT_{max}) assays*"). We also calculated the intraclass heterozygosity for each individual as
559 following: sum of heterozygous alleles / (sum of homozygous *F. aquilonia* alleles, sum of
560 heterozygous alleles, and sum of homozygous *F. polyctena* alleles).

561

562 *Identifying mtDNA origin and pairwise mtDNA mutations*

563 Using the mtDNA haplotype network, we identified the maternal species from which hybrids
564 originated (i.e., *F. aquilonia* or *F. polyctena*) as mtDNA is maternally inherited and calculated
565 pairwise mtDNA mutations as well as the pairwise geographic distances in kilometres for
566 each sample pairs. Using an Analysis of Variance (ANOVA), we tested whether the mtDNA
567 origin is associated with the latitude. Further, we correlated the pairwise mtDNA mutations
568 for the hybrid maternal lineages against the geographic distance using a linear regression to
569 test whether mtDNA mutations are correlated with latitude.

570

571 <u>Analysing long-term and cross-sectional environmental data</u>

572 *Long-term monitoring and analyses of mound activity, number of reproductive offspring, and*
573 *environmental variables between 2020-2023*

574 Between 2020 and 2023, the activity of wood ant mounds, the emergence time and number
575 of reproductive offspring (i.e. new queens and males), as well as the within-mound
576 temperature were recorded between March and June in six populations Southern Finland.
577 We observed three *F. aquilonia* populations (*Haltiala*, *Pusula*, *Soleböle*) and three *F.*
578 *aquilonia* x *F. polyctena* hybrid populations (*Bunkkerikal*, *Langholmen*, *Svanvik*).

579 The within-mound temperature was measured using HOBO data loggers (HOBO 64K
580 Pendant Temperature Data Logger, Bourne, USA). To analyse the within-mound
581 temperature, we first calculated means for each month over the three observation years for
582 hybrids and *F. aquilonia* separately. As the data was not normally distributed, we compared
583 the means using Mann-Whitney-U tests.

584 The mound activity was recorded as a binomial (1 = active; 0 = non-active). We further
585 recorded the date and counted and/or estimated the number of sexual offspring present on
586 the mound (counted with 1-20 individuals; estimated with >20). For both mound activity and
587 number of reproductive offspring, we calculated the relative percentaged (i.e., relative
588 proportions calculated over all years; count data for focal species/count data for both
589 species). We used a proportion test for the mound activity to test whether the mound activity
590 differed between hybrids and *F. aquilonia* for each month separately. We used a Pearson's
591 Chi-Squared test for count data to compare the number of estimated reproductives under the
592 null hypothesis of no difference between the species for each month separately.

593

594 *Analyses of cross-sectional environmental variables recorded during sampling*

595 During sampling, we recorded the within-mound temperature and mound volume (detailed
596 above). To assess whether hybrids and *F. aquilonia* differ in these variables in the sympatric
597 area (i.e., latitude lower than 63.99), we compared the variables separately in a linear mixed
598 effect model (LMER, lme4 package (*76*)). The explanatory variables were the genomic
599 assignment of taxa and the latitude of each sample, and the random factors were the
600 population ID and the date of sampling. The model fit was checked using dHARMa (*77*). To

601 calculate a R^2 value for each model, we created an intercept-only model for each variable
602 and included the same random effects. Specifically, we calculated the Nagelkerke Pseudo-
603 R^2 (squaredGLMM function, MuMIn package (*78*)). Such a Pseudo-R^2 value is a relative
604 measure among similar models indicating how well the model explains the data and shows
605 values for the fixed effects as well as fixed and random effects together. To check whether
606 the species differed in the variables, we calculated pairwise contrasts (emmeans, adjusting
607 for multiple testing; (*79*)). We further tested whether the mound volume is associated with
608 the latitude by correlating both in a linear regression – separately for hybrids and *F.*
609 *aquilonia*. For this, we excluded one hybrid and one *F. aquilonia* outlier mounds with
610 extremely large dimensions using z-score outlier tests for both taxa separately. We further
611 split the hybrid and *F. aquilonia* data sets into a "southern" and "northern" area based on
612 their respective latitudinal means and compared the means within taxa using a Welch's t-test
613 for hybrids and a Mann-Whitney-U-test for *F. aquilonia* (data not normally distributed).

614

615 Critical Thermal Maximum (CT_{max}) assay and analyses

616 *Conducting the CT_{max} assay*

617 From each mound, four workers were used in an CT_{max} assay using a dry bath (Eppendorf
618 block heater, Thermomixer R, Hamburg, Germany). We chose a dry bath because body size
619 does not influence heat tolerance in a dry bath assay compared to using a hot plate (*43, 80*).
620 To avoid potential experimental acclimation, we tested the ants on the same day as they
621 were sampled (*81*). Workers were transferred individually to an Eppendorf tube and sealed
622 with cotton briefly before the start of the experiment to minimise the time each worker spent
623 inside the tube. The tubes with workers were then placed in a block heater, which marked
624 the start of the experiment. Workers were exposed to 40 °C for four minutes (Fig. 1 F inset).
625 The temperature was then gradually increased to 42, 45, 47, and finally 49 °C. Each
626 temperature step was held for approximately three minutes. Between temperature steps, the
627 temperature of the block heater increased to the respective temperature in one or two
628 minutes. These steps were selected based on pilot studies. In total, the assay lasted 20
629 minutes. As a control, we transferred one worker from each population individually to a
630 single tube, sealed it with cotton, and kept it at room temperature to ensure that workers do
631 not fall into a coma and die due to other circumstances.

632 Each minute, the state of the workers inside the tubes was recorded, with workers either
633 being "active" or "knocked down". This procedure was repeated until four workers from each
634 mound were tested. A digital thermometer ("Prima", Amarell, Kreuzwertheim, Germany) was
635 placed inside a tube and sealed with cotton to measure the temperature inside the tube
636 during the assay. This temperature was recorded at the beginning of each minute. The order
637 in which the workers were placed in the dry bath was block-randomised to minimise potential
638 heating differences of the block heater. To reduce any batch effects, at least one worker
639 from each colony was tested in the same CT_{max} run.

640

641 *Survival analysis and associations with mtDNA and hybrid index*

642 To check if ants from hybrid and *F. aquilonia* populations differ in the CT_{max} assay, we
643 conducted a survival analysis. For this, the first timepoint when a worker was recorded as
644 knocked-down was selected and analysed (surv function in the survival package, (*82*);
645 plotted with survfit2 function, ggsurvfit package, (*83*)). We conducted a non-parametric
646 Kaplan-Meyer survival analysis with the state of the worker as a response variable, and the
647 time and the species/hybrid identity as explanatory variables. We then extracted the survival
648 probabilities for minutes 8-12 from the survival analysis. These minutes encompass
649 temperature values around 45 °C, which represent a biologically relevant temperature, when
650 *Formica* ants fall into heat coma (identified in a pilot study, data not shown). To account for
651 climatic changes but still compare hybrid and *F. aquilonia*, we conducted two approaches: In
652 the first approach, we only selected samples from a sympatric area (i.e., latitude lower than
653 63.9), and in the second approach, samples from entire Finland.

654 As we found hybrids that were sired from either parent, *F. aquilonia* or *F. polyctena*, we
655 tested whether the temperature at which ants fall into heat coma differs between the
656 maternal lineages using a one-sided Mann-Whitney-U test. Additionally, we tested whether
657 the hybrid index is correlated with the temperature at which ants fall into heat coma using a
658 linear regression.

659

660 <u>Hybrid persistence and expansion identified by 5-year climatic averages</u>

661 *Number and persistence of hybrids and geographic extent of hybrids*

662 In the current sampling 2023, we re-visited 42 out of 75 populations from the previous
663 sampling (*27*) that were already known to assess the number and persistence of hybrids as
664 well as the geographic extent of hybridisation. Whenever possible, workers from the same
665 mound were sampled, or workers from mounds within a distance of 500 metres, when the
666 focal known mound was abandoned. Based on the taxa (i.e., species of hybrid) identity
667 retrieved in both studies, we checked if the taxa persisted or changed. We also calculated
668 the number of *F. aquilonia x F. polyctena* hybrid samples in both data sets and checked the
669 latitude between data sets where hybrid populations were found. To check whether the
670 frequency of hybrid samples has increased over time and with latitude conducted a
671 multinomial logistic regression. For this, we lumped all cold-adapted (*F. aquilonia, F.*
672 *lugubris*) and all warm-adapted (*F. pratensis, F. rufa*) species together and used the taxxa
673 identity (i.e., hybrids as well as cold- and warm-adapted species) as the response variables
674 and the time (as factor) and latitude (as continuous variable) as explanatory variables
675 (interaction model; multinom function, nnet package (*84*)). We manually calculated a Z-score
676 (i.e., Wald Z), 2-tailed z-test, and a Nagelkerke Pseudo-R^2 value for the model values. To
677 check the model fit, we compared the model's AICc with the AIC of an intercept-only model.

678

679 *Calculation and analyses of 5-year climatic averages*

680 To calculate climatic changes over time for the sampling locations, we downloaded
681 temperature and precipitation data from the Finnish Meteorological Institute (FMI; Aalto et
682 al., 2016) and extracted the data for each mound from digital rasters (FMI ClimGrid gridded

daily climatology) at a 10-km spatial resolution for up to six years before the sampling year. We then calculated averages over 5 years before the respective sampling year. For example, if a mound from population was sampled in 2023, the climate for this mound was averaged over the years from 2018 to 2022. If it was sampled in 2005, it was averaged over the years from 1999 to 2004, etc. We calculated six climatic variables, i) winter mean temperature, ii) spring mean temperature, iii) winter mean precipitation, iv) spring mean temperature, v) the number of days above 0 °C in winter, and vi) the number of days below 0 °C in spring. Winter and spring were defined as the months of December to February and the months of April and May, respectively, and identically calculated as in Satokangas et al. (2023; (*27*)) for comparability.

For the climatic change analysis, we used the current sampling as well as only samples from 2005 originating from the previous sampling. We then defined three ranges. A ‘hybrid’ range where we found hybrids in 2005, an ‘expanded’ range at higher latitudes where we found hybrids in 2023, and an ‘expanded’ range at higher latitudes where we did not find hybrids in 2005. Our rationale was as follows: If climatic changes have allowed a northward expansion of hybrids, we expect that the climate in the expanded range has changed in a way that is favourable for hybrids. Specifically, we expect that the expanded range in 2023, where hybrids have been found, should have a similar climate compared to that of the hybrid range in 2005.

We then conducted three comparisons. i) To test whether the climate has changed and allowed a northward expansion of hybrids, we compared the climate of hybrid populations in the hybrid range found in 2005 (lower latitudes, range = 59.92 to 62.56, Fig. 2B, light purple colour; N=5; (27)) with the climate of hybrid populations in the ‘expanded’ range in 2023 at higher latitudes (latitudinal range = 62.57 to 63.97, Fig. 2 B, dark purple colour; N=4). ii) We also compared the climate of hybrid populations in the hybrid range found in 2005 (lower latitudes, range = 59.92 to 62.56, Fig. 2 B, light purple colour) with the climate of hybrid populations in the ‘expanded’ range but using climatic data from 2005 where hybrids were absent (latitudinal range = 62.57 to 63.97, Fig. 2 B, yellow colour; N=4). iii) Lastly, we compared the climate of hybrid populations in the ‘expanded’ range in 2023 (higher latitudes, range = 62.57 to 63.97, Fig. 2B, dark purple colour) with the climate of hybrid populations with the climate of hybrid populations in the ‘expanded’ range but using climatic data from 2005 (latitudinal range = 62.57 to 63.97, Fig. 2 B, yellow colour).

Hybrids with different mtDNA parental species were combined in the comparison because they did not differ in the environmental variables. Further, due to a low sample size for each range, we conducted a non-parametric bootstrap approach. For each comparison, we re-sampled the data 1,000 with replacement and calculated the difference in the means. To calculate a p-value, we extracted the 95% confidence intervals from the mean differences and counted the bootstrap values in which the mean difference was on the opposite side of zero from the observed mean, which indicates a significant difference between the data. We corrected the results for multiple testing by applying a Holm-Bonferroni correction.

We further tested whether the climate has overall changed in both hybrid and *F. aquilonia* populations over 18 years. For this, we compared the same six environmental variables described above within and between hybrid and *F. aquilonia* populations and between previous and current sampling. We compared the environmental variables using t-tests (if

normally distributed) or Mann-Whitney-U tests (if not-normally distributed) and corrected the results for multiple testing.

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Data curation: PK, PH
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Writing - Review & Editing:
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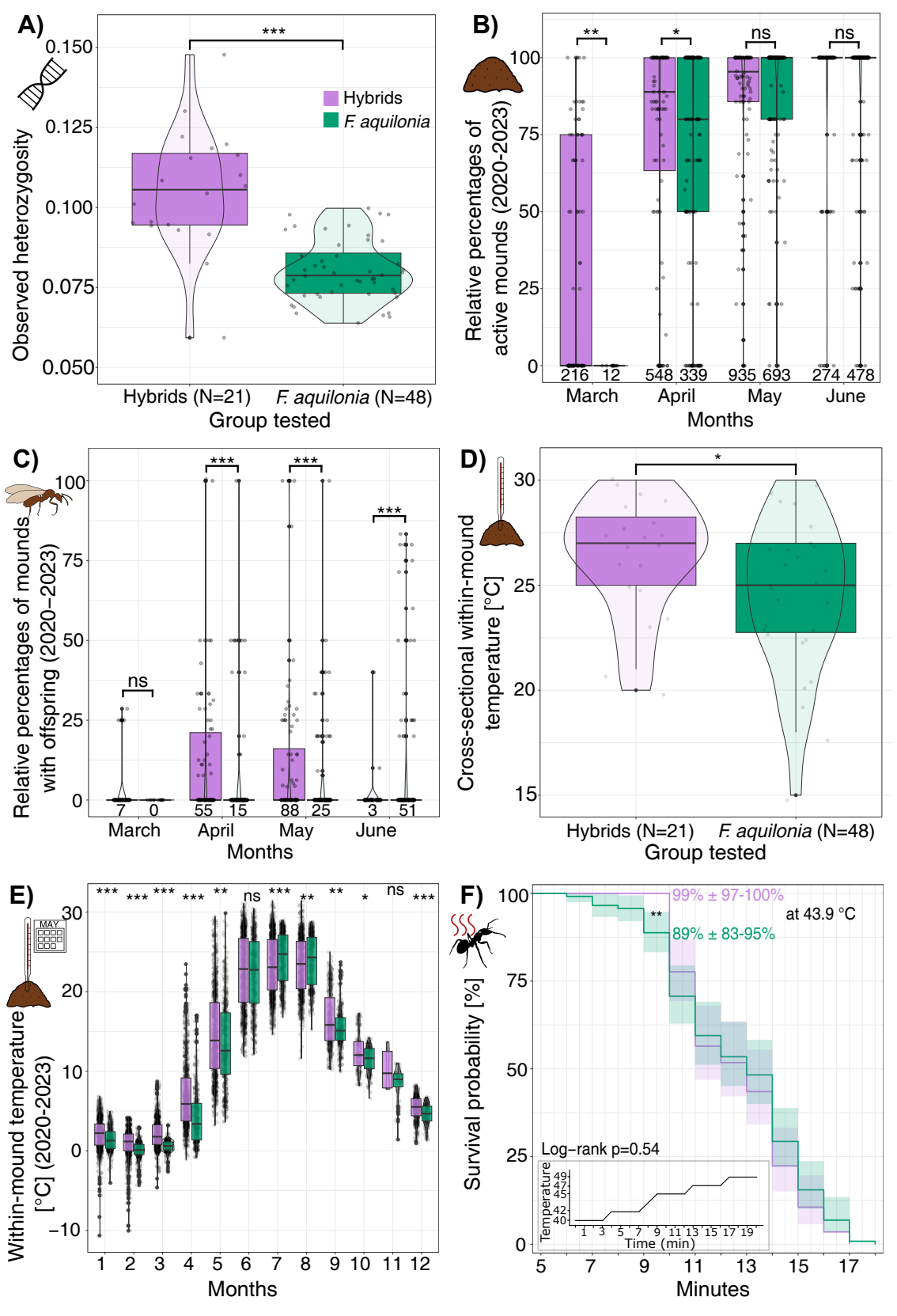
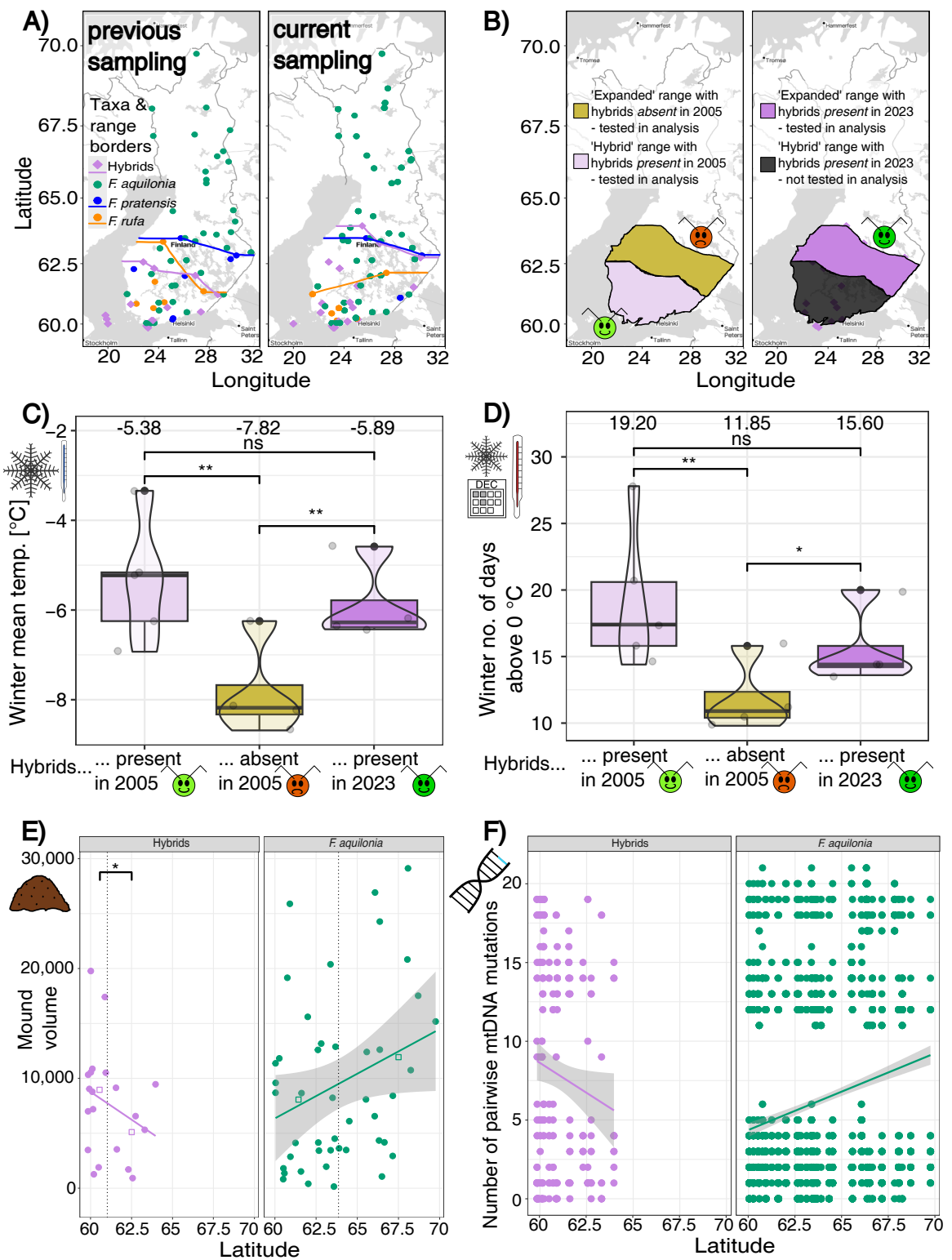

A)
Observed heterozygosity
0.150
0.125
0.100
0.075
0.050
***
Hybrids
F. aquilonia
Hybrids (N=21)
F. aquilonia (N=48)
Group tested
B)
Relative percentages of active mounds (2020-2023)
100
75
50
25
0
**
*
ns
ns
216 12
548 339
935 693
274 478
March
April
May
June
Months
C)
Relative percentages of mounds with offspring (2020–2023)
100
75
50
25
0
ns
***
***
***
7 0
55 15
88 25
3 51
March
April
May
June
Months
D)
Cross-sectional within-mound temperature [°C]
30
25
20
15
*
Hybrids (N=21)
F. aquilonia (N=48)
Group tested
E)
MAY
Within-mound temperature [°C] (2020-2023)
30
20
10
0
−10
*** *** *** *** ** ns *** ** ** * ns ***
1 2 3 4 5 6 7 8 9 10 11 12
Months
F)
Survival probability [%]
100
75
50
25
0
99% ± 97-100%
89% ± 83-95%
at 43.9 °C
**
Log−rank p=0.54
Temperature
49
47
45
42
40
1 3 5 7 9 11 13 15 17 19
Time (min)
5 7 9 11 13 15 17
Minutes


A)
previous sampling
current sampling
Taxa & range borders
Hybrids
F. aquilonia
F. pratensis
F. rufa
Finland
Helsinki
Tallinn
Stockholm
Saint Petersburg
Latitude
Longitude
B)
'Expanded' range with hybrids absent in 2005 - tested in analysis
'Hybrid' range with hybrids present in 2005 - tested in analysis
'Expanded' range with hybrids present in 2023 - tested in analysis
'Hybrid' range with hybrids present in 2023 - not tested in analysis
C)
Winter mean temp. [°C]
-5.38
-7.82
-5.89
ns
**
**
Hybrids...
... present in 2005
... absent in 2005
... present in 2023
D)
Winter no. of days above 0 °C
19.20
11.85
15.60
ns
**
*
DEC
Hybrids...
... present in 2005
... absent in 2005
... present in 2023
E)
Mound volume
Hybrids
F. aquilonia
*
Latitude
F)
Number of pairwise mtDNA mutations
Hybrids
F. aquilonia
Latitude