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Original Article

Genetic identification of *Formica rufa* group species and their putative hybrids in northern Europe

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Abstract

Wood ants of the *Formica rufa* group in northern Europe were studied by using allozyme variation with emphasis on species differences and hybridization. A total of 40 populations and 14,403 workers from 1394 nests were analyzed. Resolution of allozymes was not good enough for classifying individuals or even nest samples into species but proved useful for clustering populations and indicating potential areas of hybridization. Earlier morphological studies have shown that it is difficult to separate northern *Formica aquilonia* and *Formica polyctena* from each other. The present genetic results indicate that nearly all *F. polyctena* in Finland are admixed and their distribution is restricted to the southern parts of the country where hybrids of the two species have been found in earlier studies. In the northernmost populations close to and north of the arctic circle, the results also indicate hybridization between *F. aquilonia* and *Formica lugubris*. The results provide background information for studies assessing patterns of genome-wide selection in hybridizing *Formica* ants and understanding the effects of climate change on the geographical distribution and hybridization of species.

Key words: Allozymes, ants, Finland, Formicidae, *Formica rufa* wood ants, genetics, hybridization, Hymenoptera, introgression.

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Introduction

The mound-building wood ants of the *Formica rufa* Linnaeus, 1761 group are ecologically dominant species in northern European forests with a big impact on the ecosystems (e.g., Stockan & Robinson 2016). A large *Formica* mound can be commonly inhabited by 10^5 - 10^6 individuals (Rosengren & al. 1987) and data collected during national forest inventories suggest densities of several mounds per hectare in Finnish forests (Punttila & Kilpeläinen 2009), but the density can be locally ten times higher in forests with polydomous colonies (Seifert 2021).

In addition to their ecological significance, the *Formica rufa* group ants have become important for studies of speciation. Several closely related species are currently recognized in northern Europe (Seifert 2021). Genomic data show that the species are relatively young, to the extent that there is incomplete lineage sorting of mtDNA (mitochondrial DNA) haplotypes in some species pairs (Goropashnaya & al. 2004). Hybridization between several pairs of species has been suggested by morphological studies (Seifert 1991, Czechowski 1993, Sorvari 2006, Seifert 2021), by apparent horizontal transfer of mtDNA (Goropashnaya & al. 2004, Seifert & Goropashnaya 2004), by population allele frequency patterns (Seifert & al. 2010), and by comparing mtDNA haplotypes and nuclear microsatellite genotypes (Korczyńska & al. 2010, Kulmuni & al. 2010). In spite of apparent reticulate evolution with introgression, there are morphological and genetic differences between the species (Seifert 2021).

One particularly interesting pair of species is *Formica aquilonia* Yarrow, 1955 and *Formica polyctena* Foerster, 1850 as there are indications of strong selection affecting the genomes in their hybrids (Kulmuni & Pamilo 2014). Both species are characterized by supercoloniality as they form large polygynous (multiple queens within a nest) and polydomous (nest mounds connected by trails) colonies. Even though the two species seem to hybridize when in sympatry, they are not the closest relatives within the *Formica rufa* group. Based on mtDNA sequences, *F. polyctena* is phylogenetically close to *Formica rufa*, and *F. aquilonia* to *Formica lugubris* Zetterstedt, 1838 (Goropashnaya & al. 2004). Both *F. rufa* and *F. lugubris* have mainly monogynous, or only weakly polygynous

colonies in northern Europe (Pamilo & al. 1994). This suggests that supercoloniality has evolved separately in the two lineages or that the ancient supergene giving rise to supercoloniality (Brelsford & al. 2020) segregates differently in these taxa. *Formica aquilonia* has been suggested to have a boreo-alpine distribution in northern Eurasia (Gösswald & al. 1965, Stockan & Robinson 2016). The distribution of *Formica polyctena* is more southern but the distribution areas of the two species overlap in many places (Stockan & Robinson 2016). In northern Europe, *F. aquilonia* is the most abundant species of the *F. rufa* group, but the distribution and abundance of *F. polyctena* is less clear (Collingwood 1979, Punttila & Kilpeläinen 2009, Sorvari 2021).

Introgression between *Formica aquilonia* and *Formica polyctena* in southwestern Finland was shown by Kulmuni & al. (2010) by using both morphological and genetic data, and genetic studies further revealed geographical differences in the level of introgression (Beresford & al. 2017). Interestingly, the sexes are differently affected by hybridization and there are significant genetic differences between the sexes (Pamilo 1993, Kulmuni & al. 2010), apparently because selection on introgressed genes seems to be acting in different directions in them (Kulmuni & Pamilo 2014). Recent results of Beresford & al. (2017) and Kulmuni & al. (2020), however, indicate that the costs and benefits of introgression may change over time and likely depend on the environmental conditions. Martin-Roy & al. (2021) showed that *F. polyctena* is more heat-tolerant and *F. aquilonia* more cold-tolerant. In hybrids, putative *F. aquilonia* alleles were more abundant on cold years and *F. polyctena* alleles were more abundant on warm years suggesting balancing selection may help to keep both alleles within hybrid populations. Together, these previous data suggest outcomes of hybridization are dynamic and distribution of both hybrids and *Formica rufa* group species may change over time and with warming climate. It is plausible that *F. polyctena* is expanding its range northwards, but such a range shift may easily remain undetected when the species are morphologically and ecologically close to each other. The aim of this study is to map the geographical distribution and signs of introgression of the *F. rufa* group species in northern Europe by using genotype data. To date, this is the largest systematic study utilizing genotype data in *F. rufa* group. Previous genetic studies have focused on small geographic regions (Kulmuni & al. 2010, Seifert & al. 2010, Beresford & al. 2017), and studies covering large geographical regions have not used genetics. Furthermore, our aim is to provide a baseline for the extent of hybridization for future reference since samples for this study come from the late 1980s. This will help in assessing impacts of climate change on the distribution of these forest keystone species.

Material and methods

The ants for this study were collected in the late 1980s from 40 localities in Finland and northern Sweden (Fig. 1, Tab. 1). Worker ants for population samples were taken from nest mounds, normally within an area ranging from 5 to 25 square kilometers. Sampling of neighbouring nests was avoided, but it is likely that many mounds sampled from a single locality belonged to the same supercolony. Ants from central Europe and Ireland were used as reference. In Denmark, Germany, and Switzerland, samples were pooled from larger areas; consequently, these samples represented a geographical region rather than a local population. The data from Irish and Swiss *Formica aquilonia* and *Formica lugubris* and from Swiss *Formica paralugubris* Seifert, 1996 have been used earlier by Pamilo & al. (1992) and from *F. aquilonia* in the Finnish Site 36 by Pamilo & al. (2005).

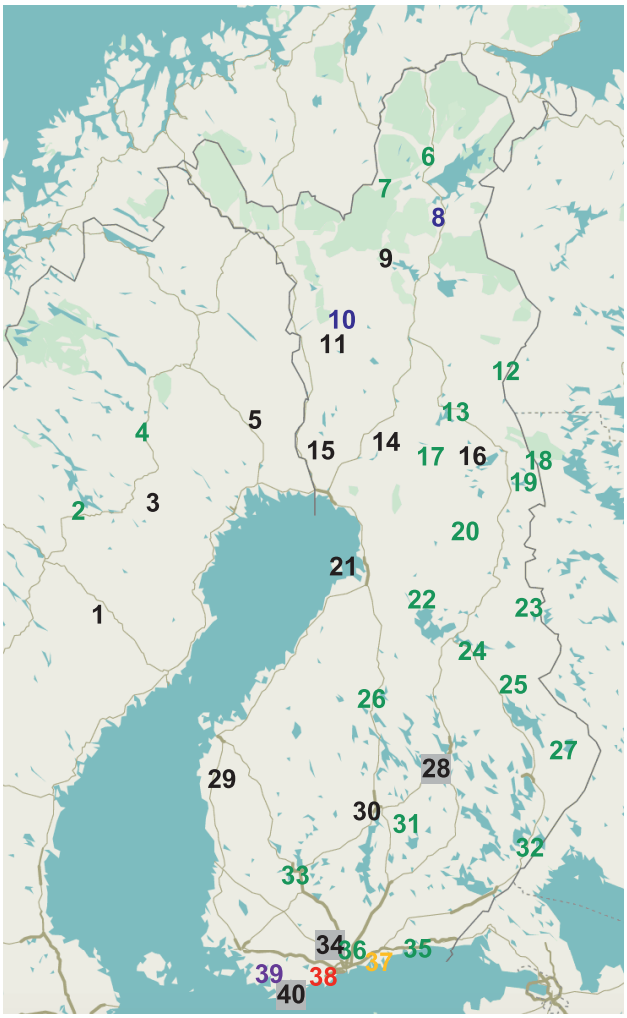

Fig. 1: The sampling locations 1 - 40 in northern Europe. Green indicates *Formica aquilonia,* blue *Formica lugubris*, black indicates a locality with both *F. aquilonia* and *F. lugubris* present, red indicates *Formica rufa*, yellow *Formica polyctena*, and violet *Formica aquilonia* × *F. polyctena* hybrid. Black with grey shading indicates a site with two or three species present in some other combinations (Tab. 1). *Formica aquilonia* × *F. polyctena* hybrid is also found in location 40. The location names are given in Table S2, and number of nests and their morphological assignment per locality is reported in Table 1.

Tab. 1: Number of nests of each morphologically identified species sampled in the localities shown in the map of Figure 1.

Location	*F. aquilonia*	*F. lugubris*	*F. rufa*	*F. polyctena*	*F. aquilonia* × *F. polyctena*
1	37	13			
2	34				
3	24	5			
4	7				
5	26	12			
6	28				
7	22				
8		19			
9	10	6			
10		14			
11	14	9			
12	40				
13	13				
14	38	7			
15	10	6			
16	23	10			
17	42				
18	36				
19	29				
20	21				
21	9	6			
22	28				
23	31				
24	31				
25	22				
26	17				
27	29				
28	29		7		
29	8	8			
30	22	6			
31	36				
32	29				
33	23				
34	56	13	6		
35	38				
36	33				
37				18	
38			24		
39					20
40		20	25		14

The species were identified at the time of collection using 5 - 10 worker ants per nest. Identification was based on the hairs at the back of the head and on the mesosoma as described by Collingwood (1979), which was the available key in the late 1980s. More precise identification criteria have been developed later (Stockan & al. 2016, Seifert 2021), but they proved hard to apply for old material that had been stored for over 35 years. Most samples were identified readily but some proved difficult. A practical problem is whether one aims to classify individuals, colonies, or populations. Individual variation exists in the morphological characters used. Possible hybridization increases the problem by producing intermediate phenotypes and by increasing morphological variation within colonies and populations. In this study, nest samples were classified, and a note was made when a sample was considered intermediate between two species, either between *Formica aquilonia* and *Formica polyctena* or between *F. aquilonia* and *Formica lugubris*.

Workers were collected from the mound surface and brought to the laboratory alive. Mostly, ten workers were genotyped from each mound. Overall, 14,403 workers from 1394 nests were genotyped.

Genotyping was done by using enzyme electrophoresis and scoring six loci (*Gpi*, *Me*, *Pep*, *Pgm*, *Est*, and *Pgk*) as described by Pamilo (1993). The genotype data was used to estimate genetic relatedness (r) of nest mates (Queller & Goodnight 1989), fixation index or the excess of homozygotes (F), and genetic distance between populations by Jost's (2008) distance D. The distances were used to cluster the populations with UPGMA (unweighted pair-group method with arithmetic mean) using MEGA version X (Kumar & al. 2018) and to explore their relationships with principal coordinates analysis (PCO) using Genalex version 6.5 (Peakall & Smouse 2006, 2012). The UPGMA dendrograms were drawn with iTOL version 5 (Letunic & Bork 2021).

Results

Six species were identified morphologically with majority being *Formica aquilonia*

Six species were morphologically identified, but as there were very few nests of *Formica pratensis* Retzius, 1783 from any one location, this species was left out from the analyses. Population samples were denoted by first two letters of the species name and the number of the locality, as shown in Figure 1 (a country code was used instead of a number for the Central European samples). A large majority (72%) of all nest samples from northern Europe (Finland and northern Sweden) were identified as *Formica aquilonia*, a total of 861 nest mounds from 32 localities (Tab. 1). The fraction of nests belonging to *Formica lugubris* was 14% (154 nests), and they were collected from various locations in Finland and Sweden, largely from the northern sampling sites. The nests of *Formica rufa* in Finland were restricted to the southern sites, and the total number sampled was 62 (5%). For genetic analyses, we used only localities where at least five nests were sampled, leading to 15 populations of *F. lugubris* and four of *F. rufa* (Tab. 1). In Finland, only one population (Site 37) was here considered to represent *Formica polyctena*. In addition, two populations (Sites 39 and 40) were morphologically close to *F. aquilonia* but came from the known hybridization area (Beresford & al. 2017) and as such were considered to represent hybrids between *F. aquilonia* and *F. polyctena*. These were denoted as hyb39 and hyb40.

We noted variation in the hairiness of samples identified as *Formica aquilonia*. In addition to the known hybridization area (Sites 39, 40), it was difficult to decide whether some individuals at Sites 25, 30, and 34 should be identified as *F. aquilonia* or *Formica polyctena*. In all these populations, a vast majority of individuals were considered to represent *F. aquilonia* with the morphological criteria used (mainly on the basis of a few hairs projecting from the occipital corners of the head). While it was difficult to distinguish some *F. aquilonia* individuals from *F. polyctena* because they had very few short hairs in the mesosoma and at the back of the head, at some other sites the individuals were more hairy than typical *F. aquilonia* and it was difficult to decide whether they represent *F. aquilonia* or *Formica lugubris*. Individuals morphologically intermediate between these two species were found at several of the northern populations (Sites 1, 3, 5, 9, 16, 17, and 22). For this reason, a small number of nests was left out from the genetic analyses. In general, the nests were classified based on the majority of individuals in the sample.

No species-specific alleles were found from the allozyme loci used

Two to six loci in each population were polymorphic with two to nine alleles per locus (Tab. S1, Appendix, as digital supplementary material to this article, at the journal's web pages). We had to pool together two alleles (Me^{100} and Me^{95}) that were earlier used to analyze family data (Pamilo 1993) because they could not be reliably scored from the population data. The total number of alleles was 30, some of them were rare. There were no clearly diagnostic alleles separating the species, and the common alleles were largely the same in each species. The mean expected heterozygosity over loci in the Finnish and Swedish populations ranged from 0.13 to 0.40, the mean per species ranging from 0.20 to 0.32 (Tabs. 2 and S2). Clear exceptions were formed by populations of *Formica lugubris* from Ireland and Switzerland with no or very little genetic variation (Tab. S1).

The mean estimate of genetic relatedness among worker nest mates was smallest in *Formica aquilonia* ($r = 0.24$). The estimates in *Formica lugubris* and *Formica rufa* were $r > 0.50$, while the single Finnish sample of *Formica polyctena* had $r = 0.40$ (Tab. 2). However, the genotype frequencies in the species other than *F. polyctena* showed an excess of homozygotes compared with the Hardy-Weinberg expectations (Tab. 2), and this elevates the relatedness estimates (Fig. 2, Spearman rank correlation between r and F in *F. aquilonia* was $r = 0.33$, $P < 0.05$).

Tab. 2: Population genetic estimates (mean and the smallest and largest estimates) in the Finnish and Swedish populations, n = the number of populations.

Species	n	Relatedness			Heterozygosity			Inbreeding		
		Min	Mean	Max	Min	Mean	Max	Min	Mean	Max
F. aquilonia	34	-0.02	0.24	0.43	0.23	0.32	0.39	0.00	0.11	0.27
F. lugubris	15	0.35	0.54	0.70	0.15	0.27	0.40	-0.04	0.11	0.31
F. rufa	4	0.56	0.65	0.72	0.13	0.24	0.30	0.01	0.16	0.32
F. polyctena	1		0.40			0.20			-0.11	

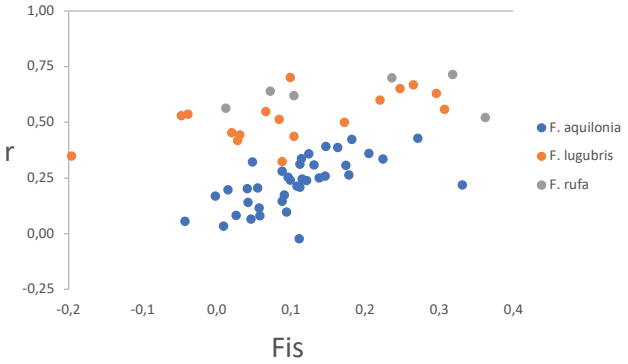

Fig. 2: A plot of the estimated fixation index F (measuring the excess of homozygosity) for the sampling locations and the estimated genetic relatedness of worker nest mates r in the Finnish and Swedish locations.

Assuming a simple model where the homozygote excess results from isolation by distance, the relatedness would be r = 0.10 in *F. aquilonia*, 0.58 in *F. rufa*, and 0.47 in *F. lugubris* under Hardy-Weinberg frequencies (i.e., when F = 0; see Pamilo 1984).

Allozyme loci classified populations into species and agreed with morphology

Clustering of individual genotypes or genotypes from single nests by various methods did not produce any clear genetic clusters because the morphologically defined species and different populations largely shared same alleles. We therefore proceeded by using populations as units in our cluster analyses.

We calculated pairwise Jost distances between the populations based on their allele frequencies. The UPGMA dendrogram drawn from this distance matrix showed some population clusters that corresponded with the morphological species identification (Fig. 3), although the dendrogram did not suggest a clear-cut separation of the species. The populations of *Formica aquilonia* formed three major clusters. Cluster A1 included all the *F. aquilonia* populations north of or close to the arctic circle (Sites 1 - 20) and three populations south of that area (Sites 22, 25, and 28). In addition, cluster A1 included a northern population of *Formica lugubris* from Site 8 (no *F. aquilonia* was collected from that site). The cluster A2 included most *F. aquilonia* populations from southern Finland as well as *F. aquilonia* and *Formica paralugubris* from Switzerland and two populations of *F. lugubris* (lu5 and lu16). The third cluster with *F. aquilonia* populations, H, included also the two populations from the putative *F. aquilonia* × *polyctena* hybridization area (hyb39 and hyb40), as well as the population from Ireland.

Most Finnish and Swedish populations of *Formica lugubris* formed a single cluster L1 in the dendrogram (Fig. 3). The exceptions were the three populations clustering with *Formica aquilonia* as explained above. Furthermore, the geographically distant populations of *F. lugubris* from Switzerland and Ireland were most different from all other populations and clustered together (L2), largely because they showed no or only very little genetic variation.

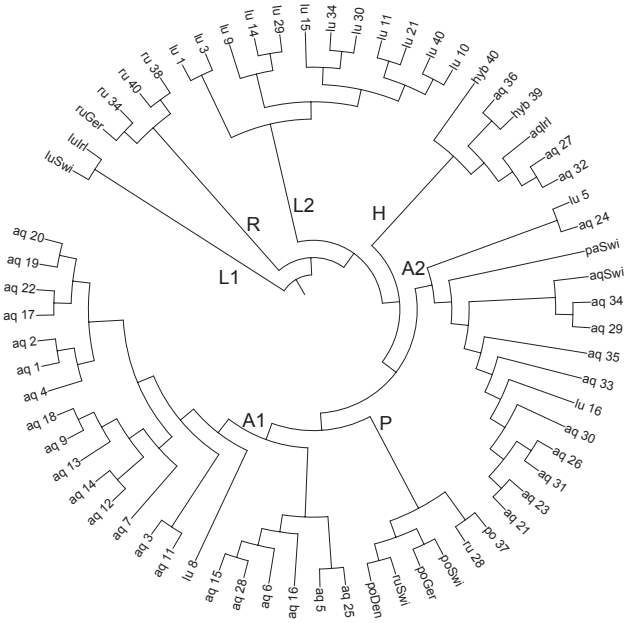

Fig. 3: Dendrogram of the populations based on the allozyme data and constructed with the unweighted pair-group method with arithmetic mean. Two first letters indicate the morphological species (aq = *aquilonia*, lu = *lugubris*, pa = *paralugubris*, po = *polyctena*, ru = *rufa*, and hyb = *aquilonia* × *polyctena*). Numbers refer to the sampling locations 1 - 40 in Finland and Sweden as shown in the map of Figure 1. Samples from other countries are indicated by the first letters of the country name (Denmark, Germany, Ireland, Switzerland).

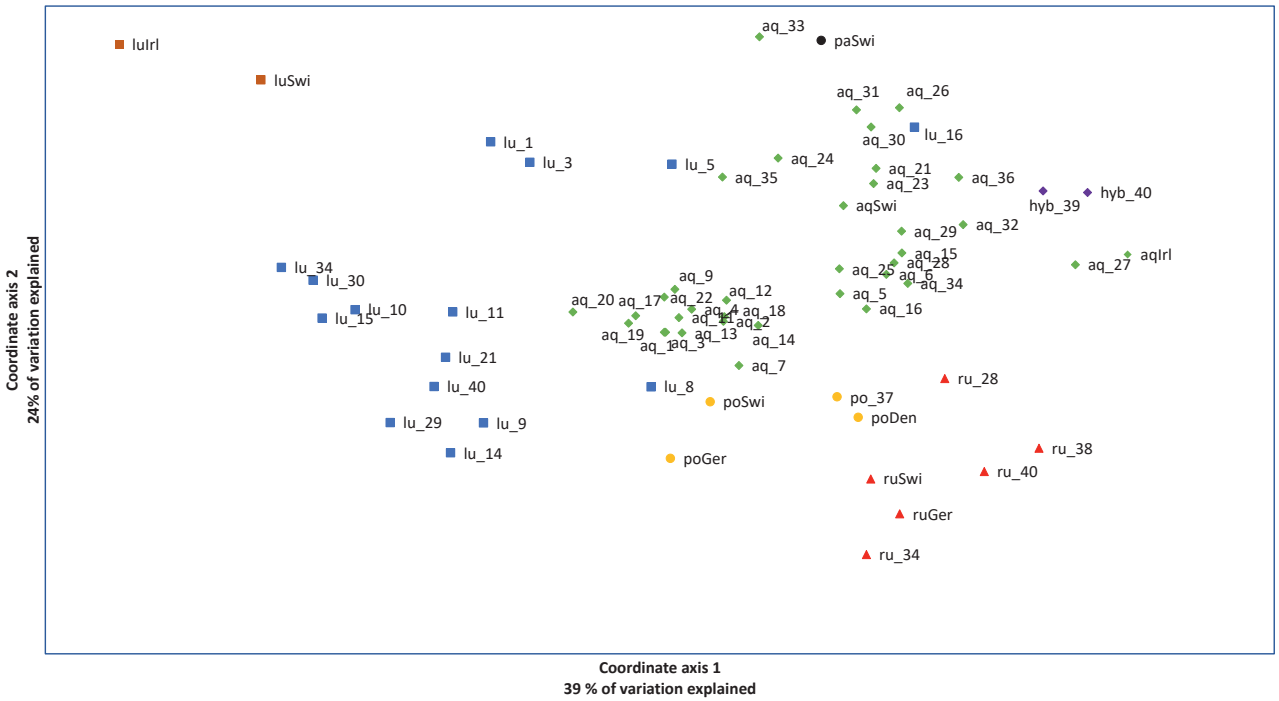

Fig. 4: Principal coordinate analysis of the population samples. Morphologically defined species are denoted as follows: *Formica aquilonia* green diamond, *Formica polyctena* yellow circle, *Formica rufa* red triangle, *Formica lugubris* (Ireland and Switzerland) brown square, *F. lugubris* (Finland and Sweden) blue square, *Formica paralugubris* black circle, putative *Formica aquilonia* × *polyctena* hybrids violet diamond.

The populations of *Formica polyctena* and *Formica rufa* formed two small clusters. Cluster P had populations of *F. polyctena* from Finland, Denmark, Germany, and Switzerland as well as *F. rufa* from Switzerland and the sample ru28 from central Finland. Cluster R had four populations of *F. rufa* (one in Germany and three in Finland).

Sensitivity of the clustering results was explored by omitting one locus at a time and redoing the UPGMA analysis (Fig S1). These new data sets led to some changes in the dendrogram, especially in places where the branching points were close to each other. Even though the details in clustering changed, the results showed that our main conclusions do not depend on a single locus. When loci are omitted from the data, the main features that we base our conclusions on remain: (a) There is some differentiation between the southern and northern populations of *Formica aquilonia*, (b) Finnish *Formica polyctena* po37 and the putative hybrid populations hyb39 and hyb40 do not cluster closely with *F. aquilonia* (i.e., clusters A1 and A2), and (c) the populations of *Formica lugubris* that clustered in either A1 or A2 (lu5, lu8, lu16) tend to still cluster with *F. aquilonia*.

The results from PCO agreed well with those obtained by UPGMA. This was expected as both analyses were based on the same distance matrix. However, the PCO results complemented the UPGMA results in an interesting way by presenting the relationships of the populations two-dimensionally. The first axis, which explained 39% of variation and separated the populations of *Formica lugubris* (both clusters L1 and L2) from all the others by placing them furthest to the left (Fig. 4). However, the populations lu5, lu8, and lu16 remained closer to or among the *Formica aquilonia* populations, similarly as in UPGMA. The second axis, which explained 24% of variation, ordered the remaining populations in such a way that the populations of *Formica polyctena* and *Formica rufa* (both clusters P and R) were separated from those of *F. aquilonia* (including also *Formica paralugubris* and the putative *F. aquilonia* × *polyctena* hybrids). Among the *F. aquilonia* populations, the samples of the cluster H were placed furthest to the right and the cluster A2 furthest up (Fig. 4). The populations of the cluster A1 were located centrally in the coordinate space.

Discussion

Clustering at the level of populations identifies species and potential admixtures

Even though the resolution of allozyme markers is not good at the level of individuals or colonies, they seem useful at the level of populations, and the obtained clusters agree to a large degree with the morphological identification. It should be reminded that the estimates of allele frequencies within populations of *Formica rufa* and *Formica lugubris* have uncertainties because of small numbers of nests and apparent monogyny of their colonies. The estimates of genetic relatedness agree with the earlier findings that the colonies of *Formica lugubris* and *F. rufa* in northern Europe are largely monogynous (Pamilo & al. 1994).

Tab. 3: Mean frequencies of some alleles within the clusters marked in the dendrogram constructed with the unweighted pair-group method with arithmetic mean (Fig. 3).

Cluster	Alleles and their frequencies within the genetic clusters										
	***Gpi*^{70}**	***Me*^{85}**	***Pep*^{70}**	***Pep*^{80}**	***Pgm*^{70}**	***Pgm*^{90}**	***Pgm*^{120}**	***Pgm*^{140}**	***Est*^{70}**	***Est*^{120}**	***Pgk*^{70}**
A1	0.17	0.03	0.00	0.55	0.19	0.00	0.02	0.01	0.02	0.21	0.26
A2	0.42	0.02	0.00	0.34	0.18	0.01	0.02	0.00	0.07	0.23	0.17
H	0.23	0.00	0.00	0.12	0.19	0.02	0.15	0.07	0.01	0.22	0.24
P	0.04	0.00	0.04	0.49	0.02	0.14	0.01	0.13	0.06	0.04	0.06
R	0.01	0.02	0.23	0.34	0.01	0.37	0.00	0.28	0.13	0.02	0.04
L2	0.29	0.10	0.00	0.83	0.04	0.00	0.01	0.00	0.00	0.08	0.36
L1	1.00	0.02	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.02	0.37

The allozyme loci used here have no clearly diagnostic alleles separating the species, and the common alleles are largely shared by all species. Closest to being diagnostic are the alleles Pep^{70}, Pgm^{90}, and Pgm^{140} that are found in *Formica polyctena* and *Formica rufa* at moderate frequencies but are practically absent in the other species. These alleles are found in different geographical areas in both clusters P and R (Tab. 3). The fact that *F. polyctena* and *F. rufa* share same alleles is consistent with earlier findings of genetically verified hybrid populations (Seifert & al. 2010) and incomplete lineage sorting of mtDNA haplotypes in these two species (Goropashnaya & al. 2004). However, the gene pools of *F. polyctena* and *F. rufa* have been found to be differentiated even when living in sympatry (Gyllenstrand & al. 2004). We had only few and small samples from these species. In Finland, *F. rufa* has a southern distribution, and only one Finnish population in the current study is classified as *F. polyctena*. The fact that the Finnish populations of these two species cluster together with central European populations of *F. rufa* and *F. polyctena* in both UPGMA (though in two separate clusters P and R) and PCO reflects the species-specific alleles separating them from the other species (see above). The populations genetically most distant from the others are *Formica lugubris* from Switzerland and Ireland. The differentiation is mainly caused by lack of or very low level of allelic variation at most loci and the fixation of the allele Gpi^{70} (Pamilo & al. 1992). *Formica lugubris* has a disjunct boreo-alpine distribution in Europe, and mtDNA haplotype sequences suggest that the Irish population may have originated from Central Europe (Mäki-Petäys & Breen 2007).

Formica polyctena occurs only in southern Finland and is admixed with *Formica aquilonia*

One aim of the study was to explore the occurrence of *F. polyctena* in Finland and its possible hybridization with *F. aquilonia*. Morphologically, we classify only one population in Southern Finland as *F. polyctena*, and it clusters together with the central European *F. polyctena* populations. It has a typical *F. polyctena* allele Pgm^{90} with a frequency (0.15) higher than in any *F. aquilonia* population, and it lacks the alleles Gpi^{70} and Est^{120} that are commonly found in *F. aquilonia*. The genetic characteristics thus agree with the morphological identification. We identified only two clear *F. aquilonia* × *F. polyctena* populations (hyb39 and hyb40). These were already known from a previous study (Beresford & al. 2017), and they fall in a separate cluster H in the dendrogram together with three other populations from south-eastern Finland (aq27, aq32, aq36), and these populations are also located furthest to the right in the PCO (Fig. 4). This points to the possibility of introgression from *F. polyctena* to *F. aquilonia* in southern Finland, even though the populations aq27, aq32, and aq36 do not belong to those with clearly intermediate morphological types. The putative hybrid cluster H shares with *F. aquilonia* several alleles that are rare in *F. polyctena* (Gpi^{70}, Pgm^{70}, Est^{120}, and Pgk^{70}). It also has an allele (Pgm^{140}) that is rare or absent in *F. aquilonia* but is found in *F. polyctena* (Tab. 3). As *F. aquilonia* is clearly much more common than *F. polyctena* in Finland (e.g., Punttila & Kilpeläinen 2009, Sorvari 2021), any backcrossing would make hybrids genetically more like *F. aquilonia*. We should also bear in mind that clustering a population in a group of putative hybrids does not necessarily mean that all the individuals or colonies in that population would be hybrids. Furthermore, the fact that the Irish population of *F. aquilonia* is included in cluster H need not suggest a hybrid status but rather reflects the effects of genetic drift in the small and isolated population (Vanhala & al. 2014).

Our results suggest that the distribution of *Formica polyctena* in Finland is restricted to the southern parts where it also has hybridized with *Formica aquilonia*. This agrees with the conclusions from morphological analyses by Seifert (2021) and a previous genetic study that found four putative *F. polyctena* populations in Southern Finland (Beresford & al. 2017). Recently, a national forest inventory suggested that a small fraction (2%) of *Formica rufa* group mounds in Finnish forests belong to *F. polyctena* and that this fraction increases slightly towards north (Punttila & Kilpeläinen 2009). Furthermore, Sorvari (2021) concluded from a large nationwide survey, that *F. polyctena* is quite common in southern and central Fin-

land up to the level of our Sampling Site 24 (about latitude 64° N, the ratio of samples identified as *F. aquilonia* to those of *F. polyctena* was 4:1 in his material) and that it is also found north of the arctic circle even close to our Sampling Site 8 (north of 68° N). Note that also Hölldobler (1960) reported *F. polyctena* in Finland slightly north of the distribution area indicated in the present study (close to our Sampling Sites 30 and 32, latitude 62° N). These previous studies were based on morphological identification.

There are alternative hypotheses for the different reports concerning the distribution of *Formica polyctena*. First, our samples were collected > 15 years before those of Punttila & Kilpeläinen (2009) and about 30 years earlier than Sorvari's (2021), and it is possible that *F. polyctena* with mainly southern distribution has benefitted from global warming and expanded its range northwards during those years. If its populations were small and scattered in the 1980s, they may have escaped our sampling. Second, hybridization between *F. polyctena* and *Formica aquilonia* may lead to difficulties in their identification and different interpretations on their distribution. After Yarrow (1955) described *F. aquilonia* as a new species, Betrem (1964) examined Karl Hölldobler's (1944) old material from Finland and Karelia (east of Finland) and concluded that most samples belonged to the newly described species *F. aquilonia*. He also noted that *F. aquilonia* in Finland is often less hairy than elsewhere and it can therefore be hard to distinguish from *F. polyctena*. Collingwood (1979) also mentioned that comparative hairlessness of *F. aquilonia* in many populations in Finland makes for confusion with the rather similar *F. polyctena*. Sorvari (2006) made morphological measurements and showed a bimodal distribution of hairiness in Finnish *F. polyctena*. He suggested that there is either large morphological variation within *F. polyctena* or the hairy type represents hybridization with *F. aquilonia*. Gene flow between species was also speculated by Pamilo & al. (1979) as that could explain patterns of both morphological and genetic variation, and introgression has been shown in genetic studies (Kulmuni & al. 2010, Beresford & al. 2017, Portinha & al. 2021). The samples here classified as *F. aquilonia* show variation in hairiness, but the genetic results of the present study do not indicate any wider recent hybridization between *F. aquilonia* and *F. polyctena* outside southernmost Finland. This agrees with the conclusions of Baroni Urbani & Collingwood (1977) and Seifert (2021) that *F. polyctena* is found only in the southernmost parts of the country (corresponding roughly to our sampling localities 34 - 40). However, if there has been ancient hybridization when colonizing northern Europe after last glaciation, most populations could be genetically admixed. In order to clarify the discrepancies between the current study and previous large-scale morphological studies and to test the hypothesis that *F. polyctena* has spread northwards with warming climate, genomic studies with recent samples are needed. Furthermore, climate change is predicted to bring previously isolated populations into contact and result in increased rates of hybridization (Pongracz & al. 2017, Scheffers & al. 2016). Introgression from warm-adapted *F. polyctena* into cold-adapted *F. aquilonia* could be beneficial with warming climate.

Genetic differentiation in *Formica aquilonia* is interesting as the southern and northern populations tend to fall in two separate clusters (Figs. 3 and 4), even though the level of differentiation is low. One possible explanation could be introgression into *F. aquilonia* from different species in the north and south (putatively from *Formica polyctena* in southern and *Formica lugubris* in northern populations), but no specific alleles suggest large-scale interspecific gene flow. It is thus likely that the slight geographic differentiation of the *F. aquilonia* populations is mainly caused by isolation by distance. Genomic studies, using both mtDNA and nuclear DNA, could reveal signs of possible historical introgression (see Portinha & al. 2021).

Morphological and allozyme data suggest *Formica aquilonia* and *Formica lugubris* hybridize in northern Finland

In addition to hybridization between *F. aquilonia* and *F. polyctena*, several populations have individuals morphologically intermediate between *F. aquilonia* and *F. lugubris*, hinting to hybridization between these two species, especially in the northern populations. Such intermediate individuals were found, for example, at Site 5, and the samples classified morphologically as *F. lugubris* (lu5) tend to genotypically cluster among or close to the *F. aquilonia* populations (Figs. 3 and 4). Other sites from which *F. lugubris* genotypically clusters among *F. aquilonia* populations are the Sites 8 and 16. The samples classified as *F. lugubris* at these sites have high estimates of genetic relatedness (r = 0.56 and 0.50, respectively) hinting to monogyny of the colonies as is the general pattern in *F. lugubris*. Site 16 has some individuals marked as atypical *F. lugubris* (i.e., less hairy than normal) in three out of 10 nests, suggesting possible hybridization history with *F. aquilonia*. Interestingly, no *F. aquilonia* was sampled at Site 8, and the *F. lugubris* individuals at this site are very hairy with no morphological indication of hybridization, yet the allozyme data clusters them together with *F. aquilonia* populations. Overall, the morphologically intermediate samples and the patterns of genetic clustering indicate that the populations of *F. aquilonia* and *F. lugubris* may have some history of past hybridization in northernmost Europe.

Conclusions

One problem in classifying the samples is whether one aims to classify individuals, colonies (nests), or populations. As mentioned above, the resolution of our genetic markers does not allow meaningful clustering of individuals, in many cases not even the nest samples. Morphological variation among individuals within a nest or among the nests within a population can represent normal variation or indicate varying levels of introgression. A genetic analysis focusing on small number of markers and populations may thus leave small amounts of intro-

gression undetected. If *Formica polyctena* is widespread in Finland, we would expect clearer signs of its presence in the genetic analyses. The current data thus indicates that the distribution of *F. polyctena* at the time of our sampling was restricted to southern Finland where it has hybridized with *Formica aquilonia*. The results also strongly indicate frequent hybridization between *F. aquilonia* and *Formica lugubris* in northernmost Europe. Distributional ranges of many insects have shifted northwards in the northern hemisphere with the on-going climate change, and the process often involves hybridization (Parmesan & al. 1999, Larson & al. 2019). Our data collected in the 1980s can serve as a reference when evaluating possible range shifts of the *Formica rufa* group species. Genome-wide data are needed to clarify the patterns of speciation, phylogeography, and hybridization history in this group of closely related *Formica* ants.

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Data accessibility

The list of populations and the population allele frequencies used in this study are available in Tables S1 and S2 as digital supplementary material to this article, at the journal's web pages.

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