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Genetic structure of native ant supercolonies varies in space and time

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Abstract

Ant supercolonies are the largest cooperative units known in nature. They consist of networks of interconnected nests with hundreds of reproductive queens, where individuals move freely between nests, cooperate across nest boundaries and show little aggression towards non-nestmates. The combination of high queen numbers and free mixing of workers, queens and brood between nests results in extremely low nestmate relatedness. In such low-relatedness societies, cooperative worker behaviour appears maladaptive because it may aid random individuals instead of relatives. Here, we provide a comprehensive picture of genetic substructure in supercolonies of the native wood ant *Formica aquilonia* using traditional population genetic as well as network analysis methods. Specifically, we test for spatial and temporal variation in genetic structure of different classes of individuals within supercolonies and analyse the role of worker movement in determining supercolony genetic networks. We find that relatedness within supercolonies is low but positive when viewed on a population level, which may be due to limited dispersal of individuals and/or ecological factors such as nest site limitation and competition against conspecifics. Genetic structure of supercolonies varied with both sample class and sampling time point, which indicates that mobility of individuals varies according to both caste and season and suggests that generalizing has to be carried out with caution in studies of supercolonial species. Overall, our analysis provides novel evidence that native wood ant supercolonies exhibit fine-scale genetic substructure, which may explain the maintenance of cooperation in these low-relatedness societies.

Keywords: ant supercolonies, cooperation, *Formica*, kin selection, microsatellites, network analysis, population genetics

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Introduction

In social insect colonies, individuals cooperate because they gain indirect fitness from helping relatives reproduce (Hamilton 1964). In a stereotypical ant nest, one or a few reproductive females (queens) produce offspring, while their sterile daughter helpers (workers) are responsible for brood care, foraging and nest defence. In these tight-knit family units, individuals share a large proportion of their genes and workers gain fitness indirectly by rearing related brood (Bourke & Franks 1995). Workers cooperate with nestmates but behave aggressively towards intruders, including foreign conspecifics. This helps the ants maintain strict boundaries between colonies and ensures workers direct their help towards relatives.

However, many ant species do not form such simple family units (Heinze 2008). In fact, from this ancestral state of family-structured colonies, ants have evolved huge variation in social structure, both inter- and intraspecifically (Bourke & Franks 1995). Kin structure

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variation is mainly caused by differences in queen number, but can also be due to variation in queen mating frequency, reproductive skew among queens and worker reproduction (Ross *et al.* 1988; MacKay *et al.* 1990; Seppä 1992; Rosengren *et al.* 1993; Sundström 1993; Bourke 1994; Baer & Boomsma 2004; Hannonen *et al.* 2004; Rheindt *et al.* 2004; Bargum *et al.* 2007; Helanterä & Sundström 2007; Seppä *et al.* 2009).

A particularly extreme form of social organization has evolved in supercolonial ants. The nests of these species can contain hundreds or even thousands of reproductive queens, and supercolonies consist of networks of interconnected nests in which individuals move freely between nests, cooperate across nest boundaries and show little or no aggression towards non-nestmates (Helanterä *et al.* 2009). Supercolonies typically originate from one or a few nests that grow by adopting daughter queens and subsequently colonize new nesting sites by budding, that is the founding of new nests by queens and workers that disperse from parental nests to new nesting sites (Hölldobler & Wilson 1977; Keller 1991). The combination of high queen numbers and free mixing of workers, queens and brood between nests results in extremely low nestmate relatedness in supercolonial ants that is often indistinguishable from zero (Holzer *et al.* 2006; Pedersen *et al.* 2006; Kümmerli & Keller 2007; Fournier *et al.* 2009).

In these low-relatedness societies, cooperative worker behaviour appears maladaptive because it may aid random individuals instead of relatives. Evolutionary theory predicts that such lineages represent evolutionary dead ends that fail to diversify and degrade eventually, for example because of lack of selection on worker traits (Linksvayer & Wade 2009) or increased selection on selfish reproductive individuals (Rankin *et al.* 2007; Helanterä *et al.* 2009). Still, supercolonial organization has evolved multiple times in ants (Helanterä *et al.* 2009) and supercolonial ants are among the most successful of all insect taxa (Savolainen & Vepsäläinen 1988, 1989; Savolainen *et al.* 1989; Wetterer *et al.* 1999; O'Dowd *et al.* 2003; Wilson 2005). This raises the question how cooperative behaviour is maintained in these systems.

A crucial step in understanding the maintenance of cooperation is a detailed assessment of the genetic structure of supercolonies. Past studies on the genetic diversity between ant supercolonies have revealed that while overall relatedness within nests is low, supercolonies can be genetically differentiated when considered on a larger geographical scale (Tsutsui & Case 2001; Pedersen *et al.* 2006; Drescher *et al.* 2007; Kümmerli & Keller 2007; van Zweden *et al.* 2007; Holzer *et al.* 2009). This indicates that processes like limited dispersal and between-supercolony competition play a role in determining the genetic substructure of populations, and gives a first indication of the importance of choosing the relevant spatial scale when assessing genetic structure of supercolonies (Helanterä *et al.* 2009). In particular, this suggests that the choice of background allele frequencies (i.e. the population wide frequencies P^* used in the relatedness estimator of Queller & Goodnight 1989) strongly affects relatedness estimates (e.g. Tsutsui & Case 2001), with higher estimates being obtained when allele frequencies from a wider sampling area are used as a comparison.

Fewer studies have addressed the genetic substructure within supercolonies. Those that do have assessed genetic structure across nests using within-nest relatedness analyses and classical measures of genetic differentiation in space such as *F*-statistics and isolation by distance, which may lack power when attempting to disentangle nonlinear spatial genetic patterns in systems with low overall relatedness (e.g. in *Formica* ants, Kümmerli & Keller 2007). In addition, studies of ant population genetics have traditionally focused on worker genotypes, which may fail to reflect the genetic reality of colonies that can be shaped by differential reproductive partitioning between worker- and gyne-producing queens (Pamilo & Seppä 1994; Bargum & Sundström 2007), and, in the case of supercolonies, between worker- and gyne-producing nests (Kennedy *et al.* 2014). Finally, high queen numbers and complex social interaction networks in supercolonial ants—which include exchange of queens, brood and workers between nests, adoption of queens by neighbouring nests and formation of new nests by budding (Helanterä *et al.* 2009)—in our opinion demands an approach tailored to these dynamics.

In this study, we test two nonexclusive hypotheses for how workers can gain inclusive fitness in these unique systems. First, nests within supercolonies may form clusters that exhibit locally elevated relatedness when viewed on a large enough spatial scale. Indeed, worker relatedness in nests of several *Formica* ant supercolonies is significantly higher than zero when relatedness is compared among supercolonies (Chapuisat *et al.* 1997; Kümmerli & Keller 2007; Holzer *et al.* 2009). Here, competition between genetically distinct supercolonies may select against intrasupercolony instability arising from selfish behaviour (Helanterä *et al.* 2009), thus contributing to the persistence of supercolonial populations (Pedersen *et al.* 2006).

Second, supercolonies may exhibit genetic variation in substructures on a temporal scale due to movement of individuals, especially workers. This is likely to be the case in temperate, hibernating species where nests within supercolonies are cut-off from each other during winter but undergo massive worker exchange in early

summer. In some temperate *Formica* ants, queens produce male and queen-destined female eggs in early spring before worker movement commences, while worker-destined eggs are produced in late spring and early summer (Bier 1952; Gösswald & Bier 1954). If workers produced the previous year hibernate in their maternal colony, they may be able to direct their help towards relatives by remaining in their natal nests until the sexual spring brood has been reared.

We use traditional population genetic methods as well as network analysis methods (network thresholding (Rozenfeld *et al.* 2008), network modularity optimization (Blondel *et al.* 2008) and maximum spanning tree analyses (Onnela *et al.* 2007)) to disentangle genetic structure of two supercolonies of the temperate wood ant *Formica aquilonia*, a species ideally suited for testing spatial and temporal components of genetic population structure because of its large, dense supercolonies (Pamilo & Rosengren 1983; Punttila 1996) and seasonal variation in brood production and worker exchange (Pamilo & Rosengren 1983; Otto 2005). We provide a comprehensive picture of genetic structure by including queens, workers and brood in our analysis and analyse relatedness across two geographical scales to test for the effect of the reference population on relatedness estimates. We investigate local genetic substructure using Bayesian and network-based clustering analyses. We also generate genetic networks based on relatedness estimates of different sample classes, allowing us to assess how genetic structure varies between groups of individuals. Finally, we test for temporal variation in genetic structure by comparing networks calculated from worker genotypes collected before and after worker exchange. Our results show clear genetic differentiation between supercolonies and demonstrate that genetic structure within supercolonies is complex, depending both on the sample class and sampling time point. More generally, our study provides a new way of resolving genetic patterns on small spatial scales using network-based methods and highlights the importance of detailed assessment of genetic structure for understanding how cooperation is maintained in social systems.

Material and methods

Study species

Formica aquilonia is a wood ant commonly found in southern Finland where it forms large supercolonies of connected nests containing hundreds of queens each (Pamilo *et al.* 2005). High queen numbers and moderate multiple mating by queens result in low within-nest relatedness in this species (Pamilo 1993; Pamilo *et al.* 2005; Sundström *et al.* 2005) and supercolonies can dominate entire ecosystems, making *F. aquilonia* an ideal model for studying the persistence of low-relatedness societies. Like other polygynous (i.e. multiple queen) ants, *F. aquilonia* is characterized by limited dispersal of queens, with new nests typically founded by groups of queens and workers dispersing on foot. Once initiated, new nests continue to grow as young queens often attempt adoption in their own or a nearby nest, so that mature nests usually contain over a hundred queens and persist over several years, or even decades. This also results in short distances between nests and high nest densities in established supercolonies (Kennedy *et al.* 2014) and may result in reduced gene flow between distant supercolonies. In our study area, distance between genetically differentiated supercolonies ranges from 200 m to 50 km (Sundström *et al.* 2005), indicating that typical dispersal distance is most likely <200 m. This is supported by the fact that median dispersal distance in *Formica exsecta*, a species that is presumed to be a stronger disperser than *F. aquilonia*, is <150 m (Vitikainen *et al.* 2015).

Sample collection

We sampled nests and recorded their location using GPS coordinates in two supercolonies near Tvärminne Zoological Station in southern Finland: supercolony Långstrand ('LA', 59.95°N/23.17°W, $n = 21$) and supercolony Myggforskogen ('MY', 59.99°N/23.23°W, $n = 20$) (Fig. 1). Our sampling was not exhaustive as *F. aquilonia* supercolonies can easily be comprised of more than a hundred nests (Kennedy *et al.* 2014). However, as we were mainly interested in genetic structure at the core of supercolonies, we located an area with high nest density (10–30 m distance between nests) at each site and sampled outwards in all directions until nest density dropped dramatically and we thus could not be sure whether nests still belonged to the same supercolony, or habitat became unsuitable. In LA, old-growth forest provided suitable habitat on both sides of the dirt road (red line, Fig. 3), allowing us to sample in a circular fashion. In MY, we were restricted to sampling in a more linear fashion as habitat was only suitable on one side of the road (black line, Fig. 4). Sampled nests covered 6.6 ha in LA and 6.1 ha in MY and the distance between two nests was 161 ± 87 m (mean ± SD, range 2.6–423 m) in LA and 155 ± 107 m (range 3.6–492 m) in MY. The two supercolonies were clearly separated from each other by distance (*c.* 5.5 km) and the presence of unsuitable habitats (e.g. water, clear-cut areas, farmland) between sites (Fig. 1). Nests were sampled once in 2010 and twice in 2011 and individuals stored in 95% ethanol at 4 °C until further analysis.

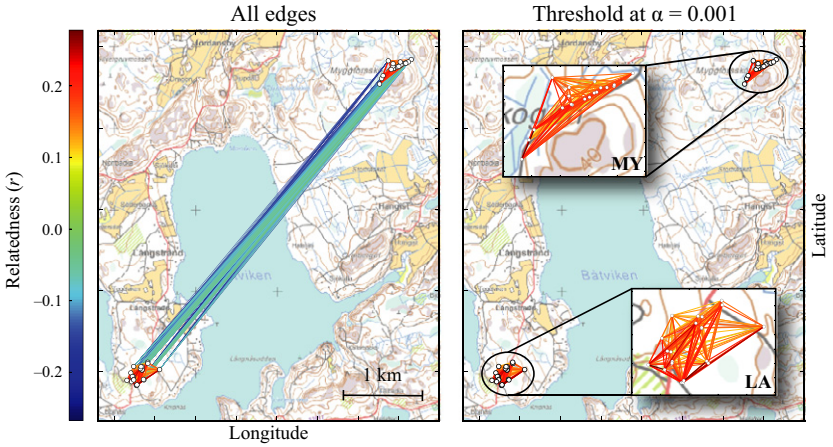

Fig. 1 Network analysis reveals positive relatedness networks within but not between two *Formica aquilonia* supercolonies. Networks were generated from GPS location data of nests in Långstrand (LA) and Myggforskogen (MY) in south-western Finland and between-nest relatedness estimates calculated from all genotypes (queens, spring workers, summer workers, brood). Significance of network connections was tested by comparing original networks (left) with randomly generated networks, and retaining only links that were significantly higher than those calculated from 1000 reference models (with $P < 0.001$, right). Colour of links reflects mean relatedness between nests. Underlying maps contain data from the National Land Survey of Finland Topographic Database 08/2015, for map key see http://www.maanmittauslaitos.fi/sites/default/files/Karttamerkkien_selitys.pdf.

In both years, we sampled once early in the season (mid-April, spring sampling) before the snow had thawed and nests were still cut off from one another. For spring sampling, we collected resident queens (i.e. mated, established queens) and spring workers from eight nests in each of the supercolonies in 2010 and from 13 (LA) and 12 (MY) nests in 2011. Nests sampled in 2011 were sampled a second time in June (summer sampling) when they contained pupae and worker exchange among nests had occurred. During summer sampling, we collected summer workers and pupae (workers, males and gynes). Our final data set contained 656 spring workers from 41 nests, 454 queens from 39 nests, 352 summer workers from 22 nests and 160 pupae from 8 nests. Data from both years were pooled prior to analyses as neither spring workers nor queens differed in mean within-nest relatedness across years (Welch two-sample *t*-test, LA—workers: $t = 0.1661$, $P = 0.8706$; LA—queens: $t = 0.721$, $P = 0.4814$; MY—workers: $t = -1.0899$, $P = 0.2956$, MY—queens: $t = -0.9946$, $P = 0.3355$; for methods see Relatedness within nests below) and estimates of pooled between-nest relatedness correlations were similar to estimates obtained when data from each year were analysed separately (Appendix S1, Supporting information; for methods see Network correlations below).

Microsatellite analysis

All samples were genotyped at eight polymorphic microsatellite loci designed for *Formica* species and tested for successful cross-amplification in *F. aquilonia* (Schultner *et al.* 2013): FE13, FE19, FE21, FE42 (Gyllenstrand *et al.* 2002); FL20, FL21 (Chapuisat 1996); FY4, FY7 (Hasegawa & Imai 2004). For DNA extraction, one leg from 8 to 16 individuals per nest and sampling class was placed in an individual well together with a 2.5:100 μL Proteinase K—Chelex solution and left to incubate overnight at 56 °C. PCRs were run in 10 μL reactions using 5 μL of QIAGEN Type-It microsatellite multiplex buffer, 3 μL of deionized water, 1 μL of optimized primer mix and 1 μL of DNA. PCR protocols were run according to QIAGEN recommendations, products analysed in 1:200 dilutions in a 3730 ABI sequencer and microsatellite peaks scored using manual bin corrections and individual peak verification in GENEMAPPER software version 4.1.

Hardy–Weinberg, linkage disequilibrium, null alleles and F-statistics

We calculated allele frequencies and mean levels of heterozygosity and tested for departures from Hardy–Weinberg using all samples (except male pupae) and loci in each supercolony separately. We tested for linkage disequilibrium (LD) between loci with log-likelihood tests in the entire data set, and within each supercolony separately. We tested for the occurrence of null alleles using ML-Null, which uses a maximum likelihood method to test for heterozygote deficiency (Kalinowski & Taper 2006). Finally, we calculated F_{st} between supercolonies using all loci, for each locus

separately, and for different combinations of loci to estimate robustness of results. Calculations were performed in GENEPOP Version 4.2 (http://genepop.curtin.edu.au).

Relatedness within nests

We calculated within-nest relatedness for each sample class and between all sample class combinations (queens and spring workers, queens and summer workers, queens and brood, spring workers and brood, summer workers and brood) with RELATEDNESS 5.0.8 following Queller & Goodnight (1989), as this represents the most widely used measure of relatedness in social insect population genetic studies, especially when relatedness within and between groups, not just among pairs of individuals, is assessed. For relatedness estimates between brood and other classes, only female pupae were included. In MY, brood was not included in the analyses as pupae genotypes were only available from two nests. For analyses within supercolonies, background allele frequencies were calculated and implemented separately for each supercolony, and nests weighted equally. We compared within-nest relatedness calculated from supercolony-specific allele frequencies with estimates obtained using allele frequencies from all samples to test for an effect of spatial scale on relatedness estimates. For all relatedness estimates, confidence intervals were calculated by jackknifing over colonies.

Network construction

To test for spatial genetic relatedness patterns both between and within supercolonies, we used network methods to construct spatial networks across supercolonies and in each supercolony separately. For network construction, relatedness within and between nests was calculated using a PYTHON implementation of (Queller & Goodnight 1989):

$$r = \frac{\sum_x \sum_k \sum_l W_x(P_y - P^*)}{\sum_x \sum_k \sum_l W_x(P_x - P^*)}$$

where W_x is a weight parameter applied for each nest, P_x is the frequency of allele l at locus k in individual x, P_y is the frequency of that same allele in the set of 'partners' of individual x, and P^* is the frequency of the allele in the population at large, with all putative relatives of individual x excluded (e.g. when calculating between-nest relatedness, the two focal nests were excluded from population allele frequencies). Background allele frequencies (P^*) were based on whole-population estimates for analyses across supercolonies and on supercolony-specific estimates for analyses within supercolonies. All nests were weighted equally with $W_x = 1$. Where male genotypes were available, they were weighed by one-half to account for haploidy. Networks of genetic structure between supercolonies were generated based on between-nest relatedness of all sample classes and nest GPS coordinates (Fig. 1, left). Networks within supercolonies were generated based on between-nest relatedness of all sample classes (Fig. 2C, D) and each sample class separately (Figs 3 and 4), and nest GPS coordinates. All PYTHON code used in network analyses is available at GITHUB (https://github.com/jsaramak/ants/); for statistical analyses such as Pearson correlations standard PYTHON packages were used (*scipy.stats.pearsonr* in SCIPY, Jones *et al.* 2001).

Detecting large scale patterns of genetic differentiation between and within supercolonies

Clustering analyses using Bayesian and network-based approaches. We analysed genetic structure between and within supercolonies using Bayesian clustering implemented in BAPS 6 (Corander *et al.* 2008) with the spatial clustering of individuals by group (i.e. nest) option. Analyses of the entire data set were run with default parameters for different maximum numbers of populations (K), where K = 2, 3, 5, 10, 25 and 50. Robustness of clusters was verified by repeating analyses with shuffled data, where individuals within supercolonies where randomly shuffled between nests while retaining the original number of individuals per nest. Within supercolonies, analyses were run for K = 2, 3, 4, 5 and 10. In all analyses, 20 iterations were run for each K. Where significant clustering was detected, we calculated pairwise F_{st} between clusters in GENEPOP Version 4.2 as described above. We also applied network-based clustering approaches, including network thresholding, modularity optimization and maximum spanning tree analyses, to networks constructed from all data except male pupae. For network thresholding, we studied the cluster structure of the joint network and the MY and LA subnetworks by progressively removing links, beginning with the lowest relatedness, and monitoring the remaining network structure. We stopped this thresholding at a stage when the network was split into two separate clusters that contained multiple nodes. For detecting clusters (i.e. modules, Fortunato 2010) in networks with modularity optimization, we applied the Louvain algorithm, which is based on partitioning the network into modules such that a quantity called *modularity* is optimized (Blondel *et al.* 2008). Modularity measures how 'unexpected' the links inside modules are with respect to a random null model. We used the weighted version of this method and applied it to full

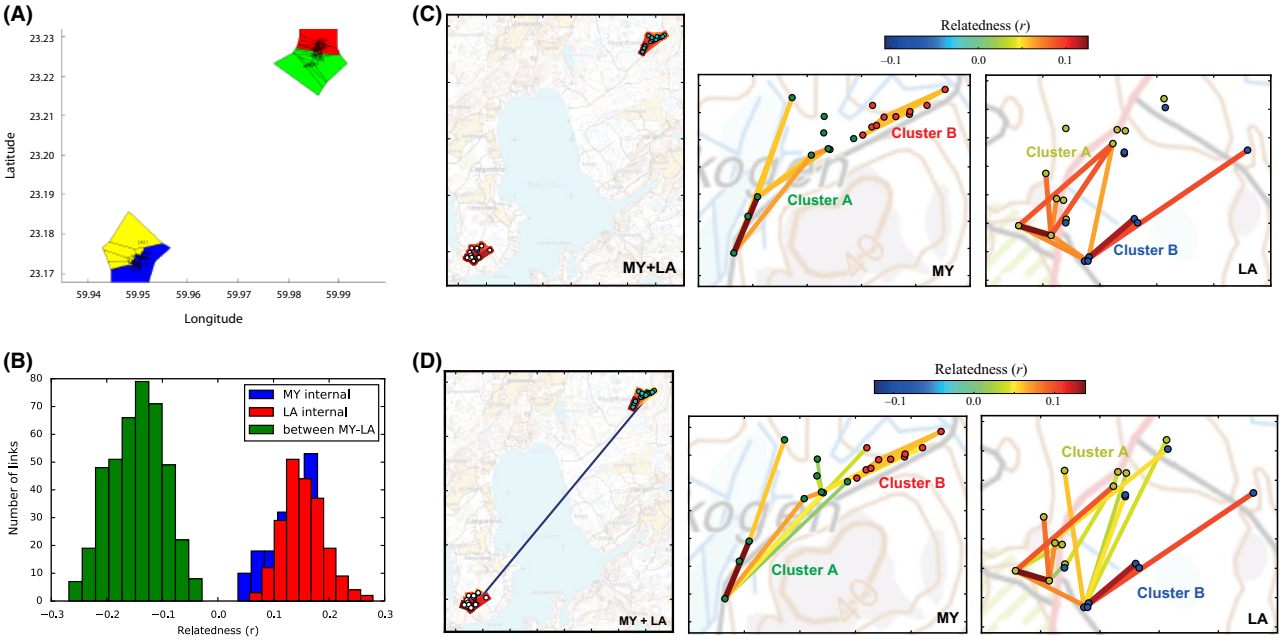

Fig. 2 Clusters detected between and within supercolonies using Bayesian and network-based approaches. (A) Clusters generated by BAPS using all data coded by nest and corresponding nest GPS coordinates. Supercolonies are clearly genetically and spatially differentiated; within each supercolony, BAPS detected two distinct genetic clusters (red/green in LA, blue/yellow in MY), but only MY subclusters were statistically robust (Appendix S4, Supporting information). (B) Relatedness links between supercolonies are clearly lower than within either MY or LA. (C) Network thresholding for the joint MY+LA network showed that the network splits into two clusters with above-threshold relatedness links (indicated by link colour) that exactly match the MY (light blue dots) and LA (white dots) supercolonies (left panel). In MY, there appear to be two clusters with above-threshold relatedness links that match well with the clusters A (green dots) and B (red dots) produced by BAPS (middle panel). Cluster structure within LA is less clear and nests with above-threshold relatedness links do not necessarily match the clusters A (light green dots) and B (blue dots) produced by BAPS (right panel). (D) The maximum spanning tree (MST) for the joint MY+LA network contained one link that connects the MY (light blue dots) and LA (white dots) supercolonies, making cluster structure between supercolonies as perfect as possible. For the MY and LA networks, there were multiple MST links between the clusters produced by BAPS (denoted by dot colour), indicating that the data cannot easily be clustered into two distinct groups.

relatedness matrices without prethresholding using the online version at http://jako.complex.cs.aalto.fi. Finally, we used maximum spanning tree (MST) analysis, which builds trees that connect all nodes of a network so that the sum of link weights is maximized. MSTs serve as a tool for network exploration, and their structure may be indicative of modules in networks (Onnela *et al.* 2003). For a perfectly modular network, the number of links of the MST that connect different modules should be as low as possible; specifically, for a network with two separate modules (defined by strong internal links and weak between-module links), the MST should only contain one between-module link, and otherwise be composed of module-internal links (see Appendix S2, Supporting information for more details).

Isolation by distance. We tested for isolation by distance within supercolonies as another way of detecting patterns of genetic substructure among nests. This was carried out in each sample class (except brood) by comparing pairwise geographical distances (in metres, converted from GPS data using R package SODA, Chambers 2008) and genetic distances between nests (based on normalized $F_{st}/(1 - F_{st})$ values calculated in GENEPOP Version 4.2, http://genepop.curtin.edu.au) using Mantel tests with 10 000 permutations (ECODIST package in R, Goslee & Urban 2007). The number of pairwise comparisons (i.e. number of nests) for each sample class was the same as for within-nest relatedness calculations (Table 1).

Detecting variation in supercolony genetic structure across sample classes and time

Random reference network thresholding. In each supercolony, we looked for significant genetic subclusters within networks calculated from different sample classes to assess whether genetic clustering of different types of individuals and sampling time points overlaps. To this end, we applied the commonly used method of

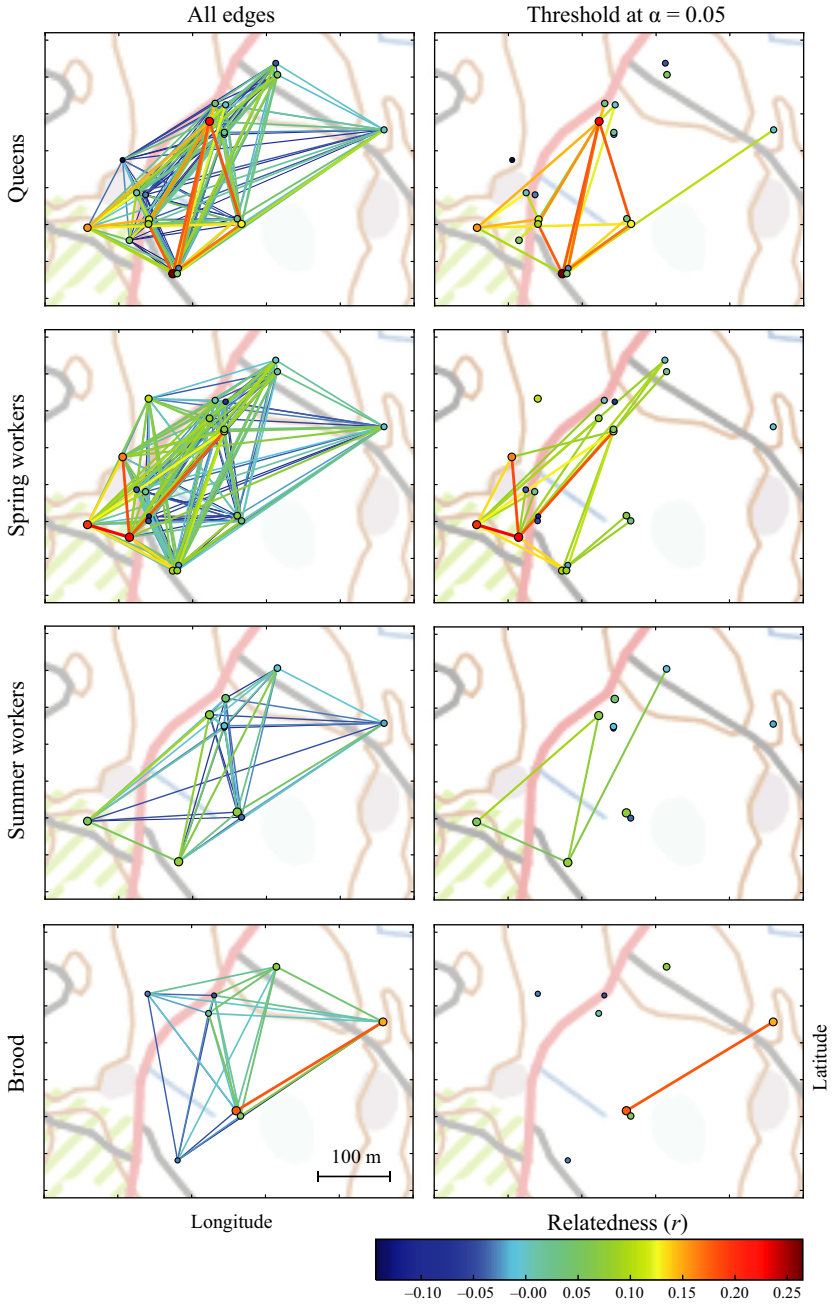

Fig. 3 Genetic networks in the LA supercolony. Networks of genetic structure were generated based on GPS location data of nests and relatedness estimates within and between nests for (i) queens, (ii) spring workers, (iii) summer workers and (iv) brood. Networks with all relatedness links are given (left) and only with links that were significantly higher than those calculated from 1000 random reference models (with $P < 0.05$, right). Differences in numbers of nests per network are due to variation in sampling. Colour of dots and links reflects mean relatedness within and between nests, respectively. Size of dots reflects the number of samples per nest, which ranged from 8 to 16 depending on sample class. Underlying maps contain data from the National Land Survey of Finland Topographic Database 08/2015, for map key see http://www.maanmittauslaitos.fi/sites/default/files/Karttamerkien_selitys.pdf.

thresholding networks (Rozenfeld *et al.* 2008) so that only the strongest links corresponding to highest pairwise relatednesses are retained. If one a priori assumes that there is cluster structure, this thresholding can be performed up to the point where the network disintegrates into clusters (Rozenfeld *et al.* 2008 and 'Clustering analyses' above). An alternative approach that does not make a priori assumptions is to use a statistical reference model to set a meaningful threshold. We used a reference model based on random reshuffling of all individuals between nests, retaining the number of ants of each class in each nest. Using such ensembles of randomized versions of observed networks for detecting nontrivial network characteristics or for statistical significance testing is common procedure in network analysis (Milo *et al.* 2002; Kivelä *et al.* 2015). We ran 1000 iterations of the null model for each network, resulting in an ensemble of 1000 reference networks in which spatial correlations were due to chance alone. We then used the distribution of all pairwise relatedness values in all networks of the reference network ensemble to set the threshold. For between-supercolony analyses, we

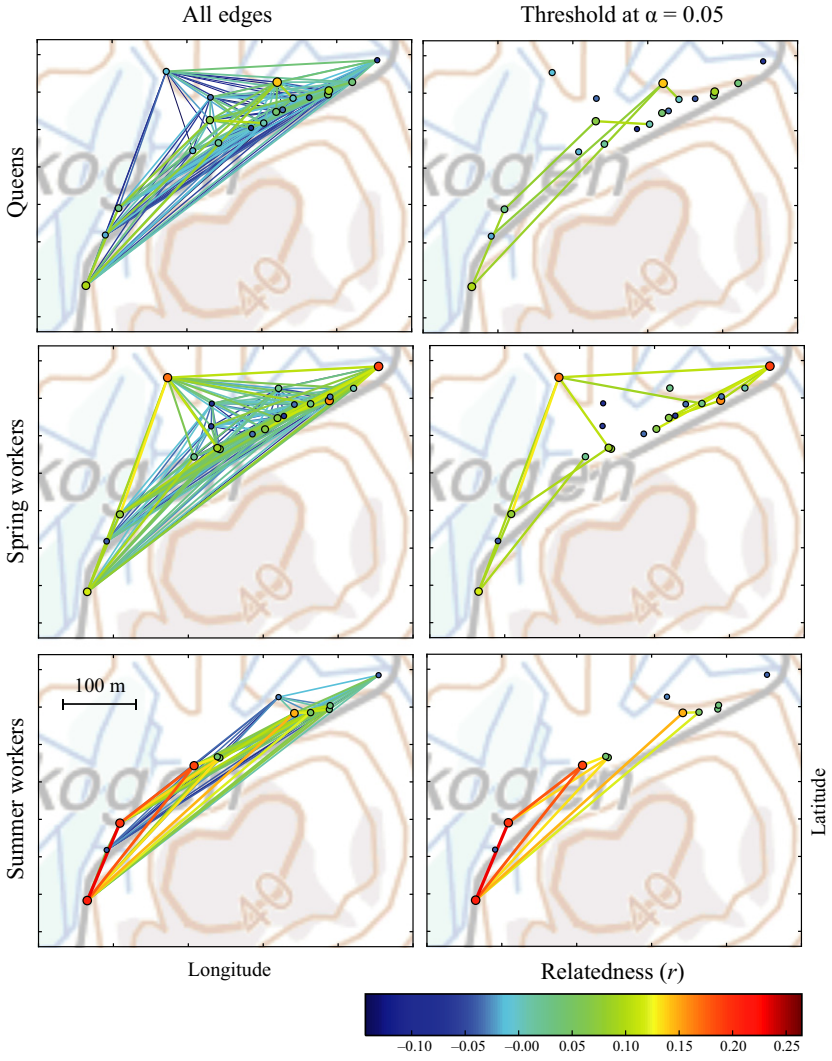

Fig. 4 Genetic networks in the MY supercolony. Networks of genetic structure were generated based on GPS location data of nests and relatedness estimates within and between nests for (i) queens, (ii) spring workers and (iii) summer workers. Networks with all relatedness links are given (left) and only with links that were significantly higher than those calculated from 1000 random reference models (with $P < 0.05$, right). Differences in numbers of nests per network are due to variation in sampling. Colour of dots and links reflects mean relatedness within and between nests, respectively. Size of dots reflects the number of samples per nest, which ranged from 8 to 16 depending on sample class. Underlying maps contain data from the National Land Survey of Finland Topographic Database 08/2015, for map key see http://www.maanmittauslaitos.fi/sites/default/files/Karttamerkkien_selitys.pdf.

retained links whose pairwise relatedness value was higher than 99.9% ($P < 0.001$) of the values in the reference ensemble, and for within-supercolony analysis, the threshold was at 95% ($P < 0.05$). The latter threshold corresponded to approximately 1.8 (1.9) standard deviations of the reference ensemble for MY (LA). The difference in the applied thresholds reflects network structure: for within-supercolony analysis, a higher threshold would not have left any links. Note that the reference ensemble is to be interpreted as a scale rather than a null hypothesis. Directly interpreting the thresholds as *P*-values for testing statistical significance can be misleading: first, links whose relatedness values do not significantly differ from the reference can still be 'real' and statistically significant, even though they are not part of the most important substructure spanned by high-relatedness links. Second, in any structured network, links are not independent, and their statistical significance should not be tested in isolation. To the best of our knowledge, there are no commonly accepted ways of testing the statistical significance of the entire outcome of thresholding networks of genetic similarity, that is computing a probability for the specific subgraph to appear when using a null hypothesis.

We visually compared the number and identity of significant links between networks calculated from the different sample classes to assess how genetic structure changes with sample class and throughout the season. When comparing links between spring and summer worker networks, we compared the number of significant links shared between spring and summer networks in respect to the total number of significant summer links (i.e. links that could potentially appear in both spring and summer networks as all nests sampled in summer were also sampled in spring).

Network correlations. We also tested for correlations between networks to assess whether genetic

Table 1 Relatedness within and between different sample classes in two *Formica aquilonia* supercolonies

Supercolony	Type	*N* nests (individuals)	$r_{supercolony}$ (95% CI)	$r_{population}$ (95% CI)
LA	All	21 (910)	**0.035** (0.02–0.05)	**0.185** (0.163–0.207)
	Queens	20 (254)	**0.045** (0.0002–0.09)	**0.201** (0.157–0.245)
	Spring workers	21 (336)	**0.043** (0.004–0.082)	**0.191** (0.152–0.229)
	Summer workers	10 (160)	0.014 (−0.024 to 0.052)	**0.162** (0.119–0.205)
	Brood	8 (160)	0.046 (−0.025 to 0.12)	**0.173** (0.098–0.249)
	Queens—spring workers	20 (254, 320)	**0.042** (0.002–0.082)	**0.197** (0.146–0.212)
	Queens—summer workers	10 (160, 160)	0.023 (−0.022 to 0.068)	**0.183** (0.141–0.225)
	Queens—brood	6 (96, 96)	**0.084** (0.034–0.137)	**0.233** (0.176–0.289)
	Spring workers—brood	7 (112, 112)	**0.072** (0.003–0.141)	**0.209** (0.153–0.265)
	Summer workers—brood	5 (80, 80)	**0.112** (0.053–0.171)	**0.261** (0.207–0.315)
MY	All	20 (746)	**0.028** (0.016–0.040)	**0.157** (0.134–0.179)
	Queens	19 (202)	0.014 (−0.014 to 0.041)	**0.134** (0.104–0.165)
	Spring workers	20 (320)	**0.039** (0.002–0.076)	**0.171** (0.133–0.209)
	Summer workers	12 (192)	**0.072** (0.013–0.131)	**0.197** (0.129–0.264)
	Queens—spring workers	19 (202, 304)	0.022 (−0.006 to 0.050)	**0.144** (0.114–0.175)
	Queens—summer workers	11 (140, 176)	**0.060** (0.004–0.116)	**0.181** (0.119–0.243)

Values in bold are significantly higher than zero as their confidence intervals do not overlap with zero.

substructure of supercolonies varied depending on sample class or year. Most importantly, we wanted to know whether within-nest relatedness of one class is correlated with within-nest relatedness estimates of the other classes across all nests, as would be expected if nests were genetically homogenous across different sample classes. Genetic homogeneity across queens, spring workers, summer workers and brood would imply that dispersal within supercolonies (i.e. budding of groups of queens and workers) and movement patterns of individual workers overlap, so that, for example, the genetic structure of nests does not change over the course of the season. This would be expected if workers remain in their natal nest throughout their lives to ensure that they direct help towards relatives. We compared pairwise within-nest relatedness of (i) queens and spring workers, (ii) queens and summer workers, (iii) queens and brood, (iv) spring workers and brood, (v) summer workers and brood and (vi) spring workers and summer workers using Pearson correlations in samples from both years separately and in pooled data. We then compared between-nest relatedness for all combinations of classes to test whether the genetic substructure of networks calculated from different groups overlaps. Here, positive correlations would indicate that relatedness between nests is similar irrespective of sample class, which would arise if for instance seasonal movement patterns of workers mirror budding patterns within supercolonies. Significance of between-nest relatedness correlations was tested using Mantel tests with 9999 permutations (ADE4 package in R, Dray & Dufour 2007) and by comparing correlations calculated from the original data with correlations based on random reshuffling of individuals between nests (1000 permutations).

Results

Hardy–Weinberg, linkage disequilibrium, null alleles and F-statistics

We found significant departures from Hardy–Weinberg equilibrium for locus FY 4 in both supercolonies, for locus FE 42 in supercolony LA and for loci FL 20 and FL 21 in supercolony MY (Appendix S3, Supporting information). Pairwise F_{st} between supercolonies calculated from data sets with and without these loci were comparable, ranging from 0.078 to 0.167 (Appendix S3, Supporting information). As locus FY4 exhibited departure from HWE in both supercolonies, we recalculated individual pairwise relatedness without FY4 in both supercolonies and then calculated the correlation between pairwise relatedness in data sets with and without this locus. Pairwise relatedness with and without FY4 was strongly positively correlated in both LA and MY, although more highly in LA ($r = 0.95$) than MY ($r = 0.77$). Although pairwise relatedness estimates with and without FY4 were not perfectly correlated in either supercolony, they were not consistently biased in either direction (Fig. S3 in Appendix S3, Supporting information). In addition, removing FY4 did not significantly change results in clustering analyses (Appendices S2 and S4, Supporting information); we therefore decided to retain locus FY4 in subsequent analyses. When analysing all samples together, we found

significant linkage disequilibrium (LD, Fisher's global test at $P < 0.01$) between FE 13 and FL 21 and FE 42 and FL 21 (Appendix S3, Supporting information). In supercolony LA, we found significant LD between FL 20 and FY 7, FE 13 and FL 21 and FL 21 and FE 21 when analysing all samples together, but not in the individual queen and spring worker data sets. In MY, we found significant LD between FE 19 and FL 20 when analysing all samples together and between FE 42 and FL 21 in spring workers. No significant LD was detected in the MY queen data set. Because analysing genetically related groups of individuals may cause apparent linkage in some cases and as the same pairs of loci were not consistently linked, this suggests that there is no physical linkage or strong disequilibrium in these loci. Significant heterozygote deficiency was found for locus FY 4 in supercolony MY, but no other null alleles were detected consistently across the entire data set (Appendix S3, Supporting information). Pairwise F_{st} between supercolonies calculated from data sets with and without FY 4 gave comparable results (with FY 4: $F_{st} = 0.153$, without FY 4 = 0.148). We therefore chose to include all loci in our analyses.

Relatedness within nests

Overall relatedness within supercolonies was close to zero when calculated from supercolony-specific allele frequencies, although confidence intervals did not overlap with zero in either supercolony. When allele frequencies from both supercolonies were used, overall mean relatedness values increased to 0.185 (95% CI: 0.163–0.207) in LA and 0.157 (95% CI: 0.134–0.179) in MY (Table 1), which corresponds to individuals within supercolonies being more closely related than cousins ($r = 0.125$), although less than half-siblings ($r = 0.25$). Within-nest relatedness of separate sample classes ranged from 0.014 to 0.072 and was significantly higher than zero in four of seven cases when calculated from supercolony-specific allele frequencies (Table 1). In LA, queens and spring workers exhibited positive relatedness, while relatedness among summer workers and brood was not significantly higher than zero. In contrast, relatedness was positive in MY spring and summer workers, but not in queens. Overall however, relatedness calculated from different groups did not differ (Kruskal–Wallis rank sum test, LA: $X^2 = 0.883$, d.f. = 3, $P = 0.8295$, MY: $X^2 = 3.6386$, d.f. = 2, $P = 0.1621$). Relatedness estimates of separate sample classes were always higher than zero when allele frequencies from both supercolonies were implemented (Table 1).

Relatedness between sample classes ranged from 0.022 to 0.112 when calculated from supercolony-specific allele frequencies and was highest between LA summer workers and LA brood (Table 1). Relatedness between queens and summer workers in LA, and queens and spring workers in MY, was not significantly different from zero, but all other classes showed positive relatedness. There was no significant variation in relatedness estimates between comparisons (Kruskal–Wallis rank sum test, LA: $X^2 = 7.7576$, d.f. = 4, $P = 0.1009$; MY: $X^2 = 1.4007$, d.f. = 1, $P = 0.2366$). Relatedness between classes was always positive when allele frequencies from both supercolonies were implemented (Table 1).

Supercolonies are genetically different

Random reference network thresholding across supercolonies revealed that relatedness between nests of different supercolonies is generally negative, while nests within supercolonies show positive, significant relatedness links (Fig. 1). Consistent clustering of nests by supercolony irrespective of input K in Bayesian clustering analyses also points towards genetic differentiation between supercolonies (Fig. 2A and Appendix S4, Supporting information), as does lower relatedness of network links between supercolonies than within (Fig. 2B). Network thresholding for the joint MY+LA network revealed a clean split into two clusters that exactly matched with the MY and LA supercolonies, with a link density of 100% inside both supercolonies at the time of this split (Fig. 2C, left). Similarly, modularity optimization using the Louvain method divided the data into two clusters that correspond perfectly to the MY and LA supercolonies (Appendix S2, Supporting information). Finally, the maximum spanning tree (MST) for the joint MY+LA network only contained one link that connects MY and LA—cluster structure between supercolonies is thus as perfect as possible (Fig. 2D, left).

Genetic structure within supercolonies varies with spatial scale, sampling class and sampling time point

Clusters within supercolonies. We found faint clustering of nests within supercolonies with BAPS, network thresholding and MST analyses (Fig. 2A,C,D), but not with modularity optimization (Appendix S2, Supporting information). In BAPS analyses, cluster membership was consistent across the range of K values in MY but not LA (Appendix S4, Supporting information). Pairwise F_{st} values between BAPS clusters within supercolonies were low ($F_{st} \sim 0.017$) compared to between supercolonies ($F_{st} \sim 0.15$) (Appendix S4, Supporting information). Network thresholding also only detected clear spatially separated subclusters in MY, which matched the clusters produced by BAPS (Fig. 2C, middle). In LA, even

though thresholding detected two clusters, many of the links between nests were weak enough to be below the threshold and network clusters did not overlap well with BAPS clusters (Fig. 2C, right). Together, this suggests that genetic differentiation is more pronounced in MY than in LA. In MST analysis, there were multiple links between clusters in both MY and LA, indicating that the data cannot easily be clustered into two distinct groups in either supercolony. However, the number of links clearly fell below random reference expectations, indicating that many node pairs in the clusters have stronger internal connections than the connections between them (see also Appendix S2, Supporting information). This result is in line with the interpretation that both MY and LA have very weak subclusters.

Isolation by distance. We did not find significant isolation by distance between nests in LA when all sample classes were analysed together (Mantel test; all samples: $r = 0.13$ (95% CI: −0.01 to 0.27), $P = 0.06$, $n = 21$ nests). In contrast, isolation by distance was highly significant in MY [Mantel test; all samples: $r = 0.41$ (95% CI: 0.24–0.59), $P < 0.001$, $n = 20$]. When classes were analysed separately, we found significant isolation by distance in spring workers of LA but not queens or summer workers [Mantel test; queens: $r = -0.02$ (−0.17 to 0.12), $P = 0.59$, $n = 20$; spring workers: $r = 0.16$ (0.02–0.30), $P = 0.03$, $n = 21$; summer workers: $r = 0.15$ (−0.12 to 0.41), $P = 0.18$, $n = 10$]. In MY, only summer workers exhibited significant isolation by distance when analysed separately [Mantel test; queens: $r = 0.08$ (−0.12 to 0.28), $P = 0.25$, $n = 19$; spring workers: $r = 0.17$ (−0.03 to 0.38), $P = 0.08$, $n = 20$; summer workers: $r = 0.40$ (0.18–0.63, $P = 0.003$, $n = 12$)].

Networks and network correlations within supercolonies. Analyses of the genetic networks within supercolonies showed that genetic structure varies with sample class and sampling time point. In LA, comparison with reference models revealed significant relatedness links between queens (27 links between 16 nests), spring workers (nine links between six nests), summer workers (three links between four nests) and brood (one link between two nests) from different nests (Fig. 3). Queen networks shared one significant link with spring worker networks, three different links with summer worker networks and no link with brood networks. Spring workers, summer workers and brood did not share any significant links. In MY, network analysis revealed eight significant relatedness links between eight nests for queens, 13 links between 11 nests for spring workers and 14 links between nine nests for summer workers (Fig. 4). Queens shared one link with spring and summer workers and spring and summer workers shared three links. Neither within-nest nor between-nest relatedness of different sample classes was significantly correlated in either supercolony, confirming that genetic structure varies among groups of individuals (Table 2). Finally, significant subnetworks calculated from separate sample classes do not overlap with subclusters detected in the entire data set using Bayesian and network-based clustering (compare Figs 2A,C,D, 3 and 4).

Discussion

Ant supercolonies are the largest cooperative units found in nature, but it remains elusive how inclusive fitness can maintain cooperation in these low-relatedness societies. Here we show complexity and variation of genetic structure within and among populations that urges caution in generalizing results obtained from single supercolonies, or from worker genotypes alone.

Supercolonies are hot spots of locally elevated relatedness

In line with other studies of wood ant supercolonies (Pamilo 1982; Chapuisat *et al.* 1997; Pamilo *et al.* 2005; Kümmerli & Keller 2007), overall relatedness in *Formica aquilonia* nests was very low but positive when calculated from supercolony-specific allele frequencies. When different classes of individual were analysed separately, within-nest relatedness was not significantly different from zero in three of seven groups, including in queens of supercolony MY. Taking into account the ambiguities in interpreting which genetic reference population correctly corresponds to the scale of competition (Queller 1994; Griffin & West 2002), we found that expanding the reference population to account for larger geographical scale led to above-zero relatedness in all sample classes. This is compatible with our hypothesis that competition among supercolonies on larger spatial scales could play a role in the maintenance of altruism in supercolonies. That supercolonies represent hot spots of locally elevated relatedness was further underscored by higher genetic differentiation between ($F_{st} = 0.15$) than within ($F_{st} \sim 0.017$) supercolonies as estimated by F_{st}, similar to previous studies estimating $F_{st} \sim 0.2$ between supercolonies in other populations (Pamilo *et al.* 2005, 2016; Vanhala *et al.* 2014).

Differences in relatedness estimates stemming from reference allele frequencies highlight the importance of choosing the relevant scale when assessing genetic structure in ant supercolonies (Kümmerli & Keller 2007). The fact that relatedness estimates increase from near-zero to between cousins and half-siblings (a range which can also be found in nonsupercolonial species,

Table 2 Correlations between networks calculated from different sample classes in two *Formica aquilonia* supercolonies

Supercolony	Type	Correlation *r*	*N*	*P*
Within-nest relatedness correlations				
LA	Queens—spring workers	0.10	20	0.70
	Queens—summer workers	0.27	10	0.45
	Queens—brood	−0.17	7	0.71
	Spring workers—brood	−0.01	8	0.97
	Summer workers—brood	−0.24	6	0.65
	Spring workers—summer workers	−0.07	10	0.84
MY	Queens—spring workers	−0.06	19	0.80
	Queens—summer workers	0.12	11	0.72
	Spring workers—summer workers	−0.06	12	0.84
Between-nest relatedness correlations				Mantel *P*/shuffled *P*
LA	Queens—spring workers	0.04	190	0.41/0.52
	Queens—summer workers	0.15	45	0.21/0.37
	Queens—brood	0.07	21	0.42/0.48
	Spring workers—brood	0.15	28	0.20/0.35
	Summer workers—brood	0.10	15	0.38/0.48
	Spring workers—summer workers	0.12	45	0.27/0.42
MY	Queens—spring workers	0.17	171	0.09/0.23
	Queens—summer workers	0.07	55	0.36/0.49
	Spring workers—summer workers	0.31	66	0.05/0.19

Mantel *P*-values were calculated in the ADE4 package in R and based on 9999 permutations. Shuffled *P*-values represent the probability of obtaining postshuffling correlation estimates as high or higher than in the original data.

Bourke & Franks 1995) when spatial scale is expanded clearly demonstrates that this is the key to understanding the maintenance of altruism in supercolonies. Spatial scale can be a major factor in determining genetic substructure because limited dispersal abilities or dispersal barriers quickly limit gene flow between nests (Pamilo *et al.* 2005). In *Formica* supercolonies, once a nest or nests have been established in a new habitat, colonies usually reproduce by budding. Because of the limited dispersal range of walking individuals, supercolonies may display high spatial genetic viscosity (Chapuisat *et al.* 1997; Holzer *et al.* 2006, 2009; Pamilo *et al.* 2016), which can be further promoted by ecological factors such as nest site limitation and competition against con- and/or heterospecifics (reviewed in Ellis & Robinson 2014). Higher levels of inbreeding in *F. aquilonia* compared to nonsupercolonial *Formica* ants give evidence for reduced dispersal in this species (Sundström *et al.* 2005).

In our data, the increase in relatedness estimates when using allele frequencies from both supercolonies indicates that restricted gene flow significantly influences genetic differentiation between supercolonies. At the same time, significant isolation by distance and consistent detection of two genetic clusters in the MY supercolony suggests that such processes may also be relevant within supercolonies. In our study, maximum distance between nests within a supercolony was 500 m, while distance between supercolonies in the study area ranges from 0.2 to 50 km (Sundström *et al.* 2005). Thus, distances between nests within single supercolonies are large enough to be potentially affected by limited dispersal in this species, contrary to previous data showing no isolation by distance (Pamilo 1982). If limited dispersal is responsible for separation of supercolony MY into two genetic clusters, we may even speculate that in the long run, reinforcement of local relatedness networks through limited dispersal could lead to separation of the two groups, and formation of separate supercolonies.

Testing this idea requires assessment of the relevant cooperative and competitive spatial scales, that is the scale where population regulation over long time periods occurs (Queller 1994; Griffin & West 2002), taking into account factors like emergence of aggression between workers from different clusters as well as dispersal ranges and colony founding success. This appears particularly complex in native *Formica* supercolonies compared to, for example, supercolonies of invasive ants, which typically lack both genetically distinct conspecific competitors and heterospecific rivals and form genetically homogenous populations across huge areas (Tsutsui *et al.* 2000; Giraud *et al.* 2002). In our system, one way to disentangle patterns may be to sample nests located between our focal supercolonies. Adding data on allele frequencies in neighbouring nests should help increase the accuracy of relatedness estimates within supercolonies (e.g. see Chapuisat *et al.*

1997; Holzer *et al.* 2009) and illuminate how allele frequencies shift differentially with local landscape features and over geographical distances. Expanding the geographical scale would also help overcome a possible lack of power in IBD analyses caused by short distances between nests, thus allowing us to better detect changes in patterns of genetic differentiation in space.

Network construction and random reference thresholding confirmed that supercolonies exhibit significant substructure on small spatial scales and that this structure is detectable even when using supercolony-specific allele frequencies. Visual comparison of networks revealed that genetic networks differ strongly between classes of individuals. Importantly, between-nest relatednesses calculated from different classes did not show significant correlations and significant subnetworks calculated from sample classes did not overlap with genetic clusters detected in the entire data set, underscoring the importance of not relying on a single sample class nor method when analysing complex social systems. Within-nest relatedness was not correlated for any combination of classes, indicating that nestmate relatedness estimates may also vary depending on sample class. Indeed, in polygynous species like *F. aquilonia*, queens within nests may contribute differentially to offspring production, which may lead to biased relatedness estimates when only one class of sample is used (Bourke *et al.* 1997; Holzer *et al.* 2008).

Worker movement affects genetic structure but relatedness alone does not determine worker distribution

One factor that likely contributes to changes in the genetic network of supercolonies is worker movement. In our analyses, spring networks shared 0% (LA, 0 of 3 links) and 21% (MY, 3 of 14 links) of significant links calculated for summer networks. If workers refrained from moving between nests, we would expect worker genetic networks of nests sampled in spring and summer of the same year to be highly similar or even identical. Accordingly, the shift in network structure between spring and summer workers shows that worker movement may be sufficient to shuffle genotypes within the supercolony, even in the course of a single season. We hypothesized that workers rear related sexual brood in their natal nest before moving to other nests. If this is the case, we would expect relatedness to be higher between spring workers, queens and brood, than between summer workers, queens and brood. While our data provide evidence for worker movement between nests because spring and summer worker networks do not overlap strongly, relatedness between spring or summer workers and queens and brood did not differ and was uniformly low. This is contrast to our second hypothesis and indicates that temporal variation in genetic substructure cannot explain the maintenance of cooperation in these systems.

Movement of individuals between nests in ant supercolonies has been suggested to mirror strategic redistribution of resources among functional units, for example in multicellular organisms (Kennedy *et al.* 2014), thus supporting the idea that ant supercolonies are in a state of evolutionary transition from individuality (separate nests) to organismality (closed network of connected nests) (McShea 2001; McShea & Changizi 2003; Bourke 2011; Pedersen 2012; Kennedy *et al.* 2014). However, to date few studies have actually investigated how transfer of individuals (and resources) affects the substructure of these cooperative units (but see Ellis *et al.* 2014; Ellis & Robinson 2015). Our study shows that worker movement plays a role in determining the genetic substructure of supercolonies, but we can only speculate as to whether genetic network patterns mirror a functional redistribution of ants (Rosengren & Fortelius 1987; Gordon *et al.* 1992; Holway & Case 2000; Heller *et al.* 2008; Ellis & Robinson 2014).

A multitude of factors likely influences spatial genetic structure in ant supercolonies

Together, conventional analysis of spatial genetic structure together with use of network construction and clustering methods provides novel evidence that native wood ant supercolonies exhibit fine-scale genetic substructures, which vary depending on sampling scale, time, sample class and population (Table 3). On the one hand, our data provide support to the idea that supercolonies are comprised of genetic subunits that arise through budding and limited dispersal (Chapuisat *et al.* 1997; Pamilo *et al.* 2005; Holzer *et al.* 2009), where locally elevated relatedness within supercolonies (relative to a larger reference population) is sufficient to ensure the maintenance of cooperation (Helanterä *et al.* 2009). On the other hand, network-based methods using supercolony-specific allele frequencies also revealed significant genetic substructure of *F. aquilonia* queens, workers and brood that cannot be explained by geographical isolation by distance alone, meaning that other factors must contribute to shaping genetic structure.

One such factor may be restriction of sexual production to one or a few nests, much like if supercolonies were networks of functional units (Cook *et al.* 2013). With new queens and males always dispersing from similar locations, genetic differentiation between nests should be largely independent of linear distance between nests. Indeed, only half of the nests contributed to offspring production in this study and

Table 3 Analyses of genetic structure used in this study

Analyses conducted in this study	Type of analysis	Biological rationale	Observed effects in this study
Comparing within-nest relatedness calculated from supercolony-specific and combined allele frequencies	Classic	Relatedness estimates should be similar if there is unrestricted gene flow between supercolonies; if gene flow is restricted, relatedness should be higher when using combined frequencies	Relatedness estimates increased when using combined allele frequencies, indicating that factors like limited dispersal or habitat unsuitability restrict gene flow between supercolonies (Table 1)
Within-nest relatedness among individuals in different sample classes within a time point	Classic	Within-nest relatedness among workers is not directly linked to inclusive fitness benefits; comparing relatedness among workers, queens and brood allows better estimates of fitness benefits	Average within-nest relatedness varied among sample classes, and sample classes differed in their relatedness to each other; however these differences were not statistically significant (Table 1)
Isolation by distance	Classic	If dispersal of individuals in a given sample class is limited by distance on the investigated scale, we predict significant correlation between genetic and geographic distance	Estimates of IBD were mainly non significant but depended strongly on sample class and supercolony, suggesting that sample classes differ in their dispersal propensity/ability and that habitat structure in addition to distance has a significant effect on genetic structure
Clustering of nests across and within supercolonies according to genetic differentiation	Bayesian clustering	Across supercolonies, individuals should cluster together irrespective of supercolony origin if there is unrestricted gene flow. Within supercolonies, no clusters should be detected if dispersal of individuals is unrestricted	Samples from the two supercolonies clustered into distinct groups, indicating that there is limited gene flow between supercolonies. Robust subclusters were also detected in one but not the other supercolony, suggesting that habitat characteristics may restrict dispersal on small spatial scales (Fig. 2A)
Clustering of nests across supercolonies according to between-nest relatedness	Network	If there is unrestricted gene flow, relatedness between nests of the same supercolony should be similar to relatedness between nests of different supercolonies	Relatedness is significantly higher between nests of the same supercolony, indicating that there is limited gene flow between supercolonies (Figs 1 and 2C,D)
Clustering of nests within supercolonies according to between-nest relatedness	Network	If dispersal of individuals within supercolonies is unrestricted, relatedness between any combination of nests should be similar	Depending on the method used, faint subclusters were detected within supercolonies, suggesting that dispersal can be restricted on small spatial scales (Fig. 2C,D)
Within-nest relatedness among individuals in the same sample classes across time points	Network	In supercolonies, movement of individuals between nests can lead to changes in genetic substructure over time	Relatedness networks of spring workers differed from summer worker networks, showing that workers move between nests (Figs 3 and 4)

Table 3 *Continued*

Analyses conducted in this study	Type of analysis	Biological rationale	Observed effects in this study
Correlation of within-nest relatedness among sample classes	Network	Positive correlations in within-nest relatedness among sample classes are predicted if all individuals within a nest are genetically homogenous, as would be the case in nests where all members are offspring of the resident queens	Within-nest relatedness estimates from different sample classes were not correlated, pointing towards high genetic diversity within nests, and differential movement among nests of individuals from different castes (Table 2)
Correlation of between-nest relatedness within and among sample classes	Network	Positive correlations would indicate that relatedness between nests is similar irrespective of sample class, which could arise if classes overlap in their movement patterns. Different correlations at different time points imply seasonal changes	Between-nest relatedness estimates were not significantly correlated among sample classes or sampling time points, suggesting that dispersal/movement patterns vary with sample class or season (Table 2)

Classic analysis of spatial genetic structure together with network analysis shows that native *Formica aquilonia* wood ant supercolonies exhibit fine-scale genetic substructure that varies depending on sampling scale, time point and sample class.

sexual reproduction is partitioned among nests in the LA supercolony (Kennedy *et al*. 2014), suggesting that reproductive partitioning plays a role in determining overall genetic patterns. Another factor, which may provide an alternative explanation for the genetic heterogeneity in MY which manifests in consistent clustering of nests into two groups, is multicolonial origin following fusion of separate, independent nests or groups of nests. This kind of process has been shown to underlie genetic substructure in *Formica exsecta* using maternally inherited mitochondrial markers (Seppä *et al*. 2012). While the two groups are currently both part of the MY supercolony, as evidenced by much lower differentiation between groups than between supercolonies, they may represent remnants of two formerly independent supercolonies. If MY but not LA resulted from such a fusion event, this may also explain the structural differences between the two supercolonies and caution against making generalizations from a single supercolony.

The most obvious conclusion from this study is perhaps that it remains exciting to study the maintenance of cooperation in ant supercolonies (Queller 1992; Lehmann *et al*. 2008). Using a combination of traditional and network-based methods, we have provided a new way of resolving genetic patterns on small spatial scales, and our analysis of worker movement provides insight into the role of nonreproductive dispersal on spatial genetic structure of supercolonies. Further understanding the social genetic dynamics of native supercolonies requires linking genetic patterns within supercolonies to social behaviour, for instance by correlating genetic differentiation with individual aggression levels as has been performed in some species (Suarez *et al*. 1999; Le Breton *et al*. 2004; Holzer *et al*. 2006). Network analysis will again prove useful in this context as an optimal tool for analysing genetic, ecological and behavioural data (Rollins *et al*. 2012; Kivelä *et al*. 2015). Ultimately, this will lead to a better understanding of the transition from individuality to organismality in social insects and, more generally, contribute to explaining social cohesion in social evolutionary transitions.

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E.S. and H.H. designed the study and conducted sampling; E.S. conducted genotyping and genetic data analysis; J.S. conducted network analysis; E.S., J.S. and H.H. wrote the manuscript.

Data accessibility

Genotype data and nest GPS coordinates are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.c682f.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Relatedness correlations between nests across sampling years.

Appendix S2 Network-based clustering.

Appendix S3 Summary of genetic data.

Appendix S4 Results of BAPS analyses.