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Unexpected absence of a multiple-queen supergene haplotype from supercolonial populations of *Formica* ants

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Abstract

Ants exhibit many complex social organization strategies. One particularly elaborate strategy is supercoloniality, in which a colony consists of many interconnected nests (= polydomy) with many queens (= polygyny). In many species of *Formica* ants, an ancient queen number supergene determines whether a colony is monogyne (= headed by single queen) or polygyne. The presence of the rearranged *P* haplotype typically leads colonies to be polygyne. However, the presence and function of this supergene polymorphism have not been examined in supercolonial populations. Here, we use genomic data from species in the *Formica rufa* group to determine whether the *P* haplotype leads to supercoloniality. In a *Formica paralugubris* population, we find that nests are polygyne despite the absence of the *P* haplotype in workers. We find spatial genetic ancestry patterns in nests consistent with supercolonial organization. Additionally, we find that the *P* haplotype is also absent in workers from supercolonial *Formica aquilonia* and *Formica aquilonia* × *polyctena* hybrid populations but is present in some *Formica polyctena* workers. We conclude that the *P* haplotype is not necessary for supercoloniality in the *Formica rufa* group, despite its long-standing association with non-supercolonial polygyny across the *Formica* genus.

Keywords: supergene, supercolony, polygyny, polydomy, *Formica*, ants

Introduction

The undeniable success of ants is in part ascribable to their variety and complexity of social organization (Bourke and Franks 1995; Hölldobler and Wilson 1990; Wilson 1971). Some colonies consist of a single nest (= monodomy), while others consist of several interconnected nests (= polydomy; Hölldobler and Wilson 1977). Colony queen number can also vary: colonies can be headed by a single queen (= monogyne) or many queens (= polygyne) (Hölldobler and Wilson 1977). Ants have evolved many social strategies multiple times (Borowiec et al. 2021; Dahan and Rabeling 2022; Hölldobler and Wilson 1990) leading to questions as to how such diverse social strategies coevolve (e.g., Favreau et al. 2018; Rubenstein et al. 2019). Linkage of traits as part of supergenes may promote coevolution: supergenes are genomic regions containing multiple functional mutations contributing to a discrete phenotypic polymorphism, with low recombination between them (Darlington and Mather 1949; Thompson and Jiggins 2014). Theory predicts that beneficial alleles should cluster together in regions of reduced recombination, such as rearrangements, to prevent maladaptive recombinants (Kirkpatrick and Barton 2006; Yeaman 2013). Additionally, modelling work shows that social polymorphisms can result from genetic linkage between dispersal ability and social traits (Mullon et al. 2018).

Recent genetic work revealed that alternative social organization strategies have evolved convergent supergenes in as many as five ant lineages (Kay et al. 2022). A comparison of the two best-studied ant supergenes in the genera *Formica* and *Solenopsis* found that they occupy non-homologous regions of the genome (Purcell et al. 2014). Additionally, the *Solenopsis* and *Formica* supergenes have different estimated ages, both much more recent than the divergence time between the two genera—the *Formica* supergene is ancient (approximately 30 million years old; Purcell et al. 2021) and evolved once in the common ancestor of extant *Formica*, while the *Solenopsis* supergene is relatively young (approximately 1.25 million years old; Helleu et al. 2022; Yan et al. 2020).

In *Formica* ants, workers in polygyne colonies generally have at least one *P* haplotype, while workers in monogyne colonies are almost exclusively homozygous for the *M* haplotype (e.g., Purcell et al. 2014; Brelsford et al. 2020; Lagunas-Robles et al. 2021; Pierce et al. 2022; Scarparo et al. 2023; but see McGuire et al. 2022). Intriguingly, *Formica truncorum* and *Formica exsecta* have the queen number supergene (Brelsford et al. 2020) but can also exhibit a social strategy known as supercoloniality (Elias et al. 2005; Rosengren et al. 1985; Seppä et al. 2012). This derived strategy has evolved independently in several ant lineages (Helanterä 2022), including *Formica* ants (Seifert 2018). Supercoloniality is

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an exaggerated form of polydomous, polygynous social organization with genetic connectivity and resource sharing between neighbouring nests (Debout et al. 2007; Gordon and Heller 2012; Helanterä 2022). This allows for a colony to be interconnected over a broad geographic distribution. Supercolonial species exhibit a similar suite of traits classified under polygyne syndrome (Keller 1993, 1995), such as dispersal by budding, local mating, and local queen recruitment. While it is important to note that not all polydomous colonies are polygynous (Debout et al. 2007; Hölldobler and Wilson 1977), it has been suggested that stable polygyne nests have the potential to become "incipient supercolonies" given favourable ecological conditions (Helanterä et al. 2009; Huszár et al. 2014; Pedersen et al. 2006).

Supercoloniality was inferred to have multiple origins in *Formica* ants (e.g., *Formica rufa* group, *Formica exsecta* group, *Formica uralensis* group, *Formica integra* group, *Formica fusca* group; Borowiec et al. 2021). The species in the *F. rufa* group serve as an excellent model to examine the evolution of supercoloniality as the clade is socially polymorphic with several species that can form supercolonies (Seifert 2016, 2021). Of particular interest within the *F. rufa* group is the *F. lugubris* species complex (Seifert 2021). This species complex includes *Formica paralugubris* (Seifert 1996) which is characterized by its polygynous and supercolonial nature. Prior work in the Swiss Alps showed that *F. paralugubris* forms small neighbouring supercolonies (Chapuisat et al. 1997; Holzer et al. 2009), with a notable lack of aggression between workers from the neighbouring nests (Chapuisat et al. 2005; Holzer et al. 2006). While many large supercolonies typically exhibit near zero relatedness and show aggression to members of neighbouring supercolonies (e.g., Elias et al. 2005; Pamilo et al. 2005; Pedersen et al. 2006; Thomas et al. 2006), *F. paralugubris* is tolerant of non-nestmates and does not show behavioural supercolony boundaries (Holzer et al. 2006). Despite numerous studies on supercolonies in both invasive (e.g., Giraud et al. 2002; Lenoir et al. 2016; Sorger et al. 2017; Sunamura et al. 2009; Van Wilgenburg et al. 2010) and native contexts (e.g., Elias et al. 2005; Hakala et al. 2020; Holzer et al. 2009; Wiezik et al. 2017), the genetic mechanism by which supercoloniality is determined remains elusive (Helanterä 2022).

In this study, we examine the potential link between an ancient queen number supergene and supercoloniality in three species from the *F. rufa* group (Figure 1). We hypothesize that if supercoloniality is an extension of polygyny, as has been shown in some species (e.g., Huszár et al. 2014; Pedersen et al. 2006), then the polygyne-associated *P* haplotype should be found in supercolonial populations of *Formica* ants. We assess the potential for a supercolonial *F. paralugubris* population by examining the spatial distribution of genetic ancestry within nests. If multiple supercolonies are present in the population, we would expect to find spatially restricted ancestry groups each spanning multiple nearby nests within a supercolony, and little admixture between supercolonies. We then assess the generality of our findings by reanalyzing published genomic data from two additional species in the *F. rufa* group (*F. aquilonia* and *F. polyctena*) from various supercolonial populations. If there is a similar genetic underpinning to supercoloniality in the *Formica rufa* group, we would expect our findings in *F. paraluguburis* to extend to *F. aquilonia* and *F. polyctena* workers from different supercolonies.

Materials and methods

Nest sample collection and library preparation

In August 2018 and 2021, we collected worker ants from 41 total *F. rufa* group nests in the valley adjacent to Bosco Gurin, Ticino, Switzerland (46.3164° N, 8.4927° E). One nest was sampled in both 2018 and 2021. We recorded nest locations using a Garmin eTrex GPS unit.

We extracted DNA from up to five workers from each of five nests collected in 2018. The DNA was extracted with the following steps: we manually ground the head and thorax of each ant in liquid nitrogen, then followed the Qiagen DNEasy insect tissue protocol for genomic DNA extraction. We eluted in 30 µl of AE buffer. The samples were then prepared for double-digest restriction-site-associated DNA (ddRAD) sequencing using the Brelsford et al. (2016) protocol, which incorporates elements proposed by Parchman et al. (2012) and Peterson et al. (2012), with restriction enzymes EcoRI and MseI. We sent the pooled library to Novogene for sequencing on a partial HiSeq X Ten lane with 150bp paired-ended reads.

We extracted DNA from workers from 37 nests collected in 2021, for up to 5 workers per colony, by manually grinding the head and thorax in liquid nitrogen and digesting the tissue overnight at 56 °C in 180 µl ATL buffer and 20 µl proteinase K. We transferred the supernatant into deep-well plates and completed the DNA extraction with the QIAcube HT extraction robot following the manufacturer's protocol for the QiaAmp 96 DNA kit. We eluted in 100 µl of elution buffer (10 mM Tris, pH 8.0). We prepared the genomic DNA for ddRAD

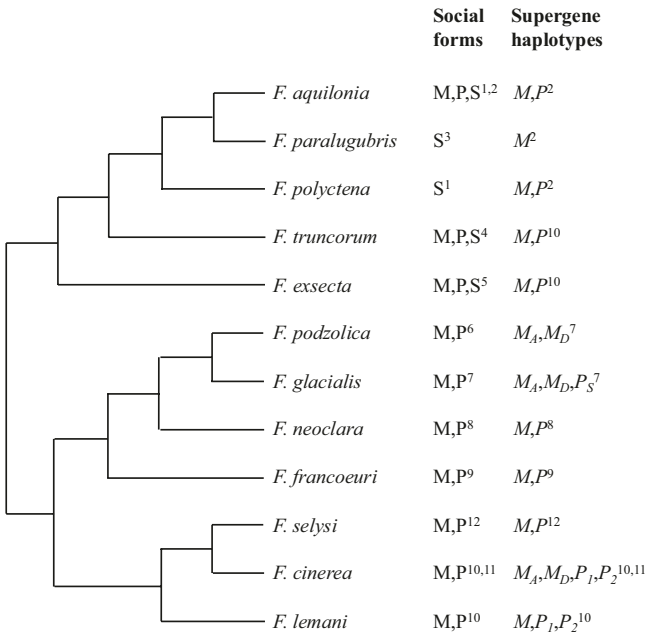

Figure 1. A phylogeny of *Formica* species with known supergene haplotypes and social forms. The tree is based on phylogenetic relationships established in Borowiec et al. (2021), Purcell et al. (2021), and Sigeman et al. (2024). The known social forms for each species are provided. Social forms are denoted as monogynous (*M*), polygynous (*P*), and supercolonial (*S*). Supergene haplotypes described in various studies are denoted as follows: monogyne-associated (M, M_A, M_D) and polygyne-associated (P, P_1, P_2, P_S). References cited: 1: Helanterä (2022); 2: this study; 3: Seifert (1996); 4: Elias et al. (2005); 5: Seppä et al. (2012); 6: Deslippe and Savolainen (1995); 7: Lagunas-Robles et al. (2021); 8: McGuire et al. (2022); 9: Pierce et al. (2022); 10: Brelsford et al. (2020); 11: Scarparo et al. (2023); 12: Purcell et al. (2014).

sequencing as described above for the 2018 colonies. We sent the pooled library to University of California San Diego's Institute for Genomic Medicine for sequencing on a partial NovaSeq 6000 sequencing lane with 150 bp paired-end reads.

Variant calling and filtering

We demultiplexed the raw ddRAD sequence data using *process_radtags* in *Stacks* version 2.60 (Catchen et al. 2013). We merged overlapping pair-end reads with *PEAR* version 0.9.11 (Zhang et al. 2014) and then aligned the reads to a reference genome of a hybrid *F. aquilonia* × *polyctena* male (Nouhaud et al. 2022a, GenBank GCA_907163055.1) with *bwa-mem2* version 2.2.1 (Vasimuddin et al. 2019), using *Samtools* version 1.16 (Li et al. 2009) to sort and merge bam files. Separately, we retrieved whole-genome sequence data from NCBI for two *Formica truncorum* males to leverage the known supergene genotypes for these samples for our analyses (Brelsford et al. 2020, Table S1). For these whole-genome samples, we used PEAR version 0.9.11 with the flag -k to retain read orientation and aligned the reads to the *Formica aquilonia* × *polyctena* reference genome (Nouhaud et al. 2022a, GenBank GCA_907163055.1) using *bwa-mem2* version 2.2.1. Then, for the whole-genome bam files, we used *fixmate* to fill mate coordinates and marked and removed PCR duplicates using *markdup* in *Samtools* version 1.16. We used *Samtools* version 1.16 (Li et al. 2009) to sort and merge the whole-genome bam files. We called genomic variants for the whole-genome and ddRAD samples together with reads that had mapping quality greater than 20 (--min-MQ 20) with *mpileup* and *call* in *bcftools* version 1.16 (Li 2011).

We filtered for high-quality SNPs to create a multi-species VCF file using the following parameters in *VCFtools* version 0.1.17 (Danecek et al. 2011). We removed indels (--remove-indels) and retained bi-allelic SNPs (--max-alleles 2) with a minimum depth of 8 (--minDP 8), minor allele count of at least 2 (--mac 2) and retained SNPs with greater than 75% presence among individuals (--max-missing 0.75). After filtering the genome-wide multi-species VCF file for high-quality SNPs, we retained any individuals that had a genome-wide SNP average depth greater 8 as calculated by --depth in *VCFtools* version 0.1.17 (Danecek et al. 2011). We used the same filtering parameters to create species-specific VCF files. Hereafter, scaffolds will be referred to as chromosomes since the reference assembly is anchored to the *Formica selysi* chromosome-level assembly (Brelsford et al. 2020, GCA_009859135.1).

Species identification

We performed a principal component analysis (PCA) in *PLINK* version 1.90b6.25 (Purcell et al. 2007), excluding chromosome 3 (--not-chr Scaffold03) since recombination is reduced on the social supergene and would create genetic structure independent of species. This analysis resulted in three distinct clusters. We sent raw reads from three representatives of each cluster to Guillaume Lavanchy, who conducted a cluster analysis combining these samples with additional ddRAD data from seven morphologically identified *F. rufa* group species (G. Lavanchy and T. Schwander, unpublished data). The inferred species identities for our three genetic clusters were *Formica paralugubris*, *Formica aquilonia*, and *Formica truncorum* (Figure 2A). In total, including the whole-genome resequencing data, we had genomic data for 23 *F. aquilonia*, 155 *F. paralugubris*, and 15 *F. truncorum* (Supplementary Table S1). Of the 41 nests we sampled in the single locality, we found the overwhelming majority were *F. paralugubris* ($n = 34$), and a smaller number were *F. aquilonia* ($n = 4$) and *F. truncorum* ($n = 3$).

Supergene genotypes

With our filtered dataset, we conducted multiple PCAs in *PLINK* version 1.90b6.25 (Purcell et al. 2007) to identify the presence of supergene genotypes in our dataset. We first conducted single-species PCAs on each chromosome for *F. paralugubris* using the species-specific VCFs. We then conducted a PCA on chromosome 3 for all species together and included two *F. truncorum* males with known supergene haplotypes to serve as positive controls in the PCA (Brelsford et al. 2020).

We also performed PCAs in *PLINK* version 1.90b6.25 (Purcell et al. 2007) in 750 kb windows on each chromosome to scan for supergene polymorphism on other chromosomes. For each individual, we plotted PC1 against the midpoint of each window. To have the PC1 axis oriented in the same direction for all windows, we transformed PC1 values when the mean PC1 value for an *F. truncorum* window was greater than the mean PC1 for the corresponding *F. paralugubris* window. We multiplied all PC1 values by −1 for windows that met these criteria.

To identify supergene heterozygotes, we calculated heterozygosity (--het) for chromosome 3 in *VCFtools* version 0.1.17 (Danecek et al. 2011). To measure the genetic distance from the reference genome, we extracted genotype calls with using "--012" in *VCFtools* version 0.1.17 (Danecek et al. 2011). In 012 format, 0 matches the reference allele, 1 is heterozygous, and 2 is homozygous for the non-reference allele. We calculated the frequency of non-reference alleles averaged across chromosome 3 for each individual (excluding missing genotypes, which are coded as −1 in this format) and converted the average to a proportion by dividing by 2.

Estimating relatedness to infer nest social form

In order to infer social form, we estimated intra-nest relatedness for each nest using *COANCESTRY* version 1.0.1.10 (Wang 2011), after extracting genotype calls in 012 format using *VCFtools* version 0.1.17 (Danecek et al. 2011) for each species. We only included nests that had at least four sequenced workers for a total of 36 nests and excluded chromosome 3 from the analysis. We excluded self-comparisons from all downstream analyses. We classified nest social form using intra-nest pairwise relatedness comparisons as determined by the Wang estimator (Wang 2002) and evaluated the values against theoretical expectations. The theoretical relatedness for workers in monandrous monogyne colony is 0.75, where workers are full sisters; in a nest headed by a doubly or triply mated queen, some pairs of workers would be half sisters with expected relatedness of 0.25 (Hamilton 1964). We expect relatedness estimates to be downward biased but more precise in reduced representation datasets compared to microsatellite-based datasets (Attard et al. 2018). To account for this downward bias, we evaluated the distribution of the pairwise intra-nest relatedness to determine what values consistently differentiated full siblings (determined via monogyne colonies) from less related pairs (Figure 3). The lack of a bimodal distribution in all *F. truncorum* nests and a single *F. aquilonia* nest were consistent with relatedness expected in full-sibling relationships in a monandrous monogyne nest. The bimodal distribution in two *F. aquilonia* nests were consistent with relatedness between pairs of half siblings and pairs of full siblings in polyandrous monogyne nests. To

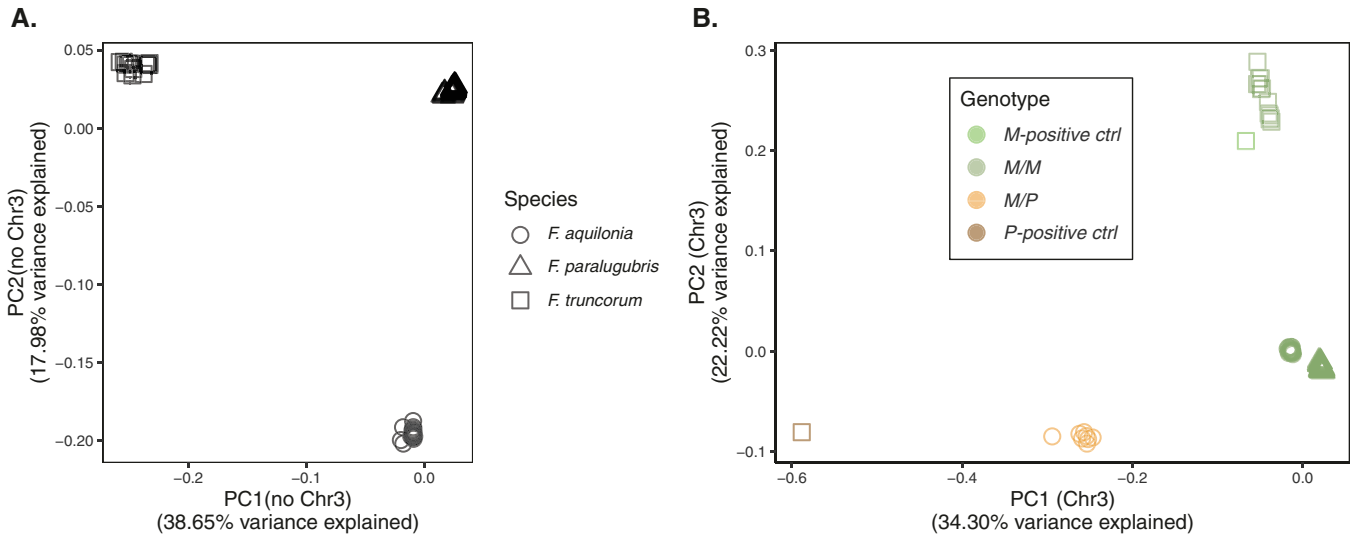

Figure 2. Principal component analysis (PCA) reveals that the majority of individuals in the study are *M*/*M* at the chromosome 3 supergene. Each point represents an individual. The individual's species is represented by its shape. The individual's haplotype (haploid males) or genotype (diploid workers) is indicated by the colour. We included two whole-genome resequencing samples with known supergene genotypes for *F. truncorum* (an *M* male and a *P* male) to serve as positive controls. (A) Using PCA of all SNPs excluding chromosome 3, we confirmed the presence of three species in our dataset: *F. aquilonia*, *F. paralugubris*, and *F. truncorum*. (B) Individuals are separated into three main clusters along PC1 for chromosome 3. The single *F. truncorum* haploid *P* male was at the extreme negative end of PC1. The homo-/hemi-zygous *M* individuals were at the extreme positive end of PC1 and grouped with the *F. truncorum* haploid *M* male in PC1 space. The intermediate cluster in PC1 space consisted of *F. aquilonia M*/*P* heterozygous workers. Notably, all the *F. paralugubris* were *M*/*M* on the supergene.

account for a downward bias, we placed the lower boundary of full sibling relationships at 0.55, which was slightly below our lowest inferred full sibling pairwise comparison. We placed the upper boundary of an unrelated pairwise comparison at 0.19 to account for high inbreeding expected in highly polygynous populations. We called nests with all pairwise relationships ≥0.55 as monogyne monandrous, nests with bimodal distribution of pairwise relationships with at least 20% ≥0.55, but none ≤0.19 as monogyne polyandrous. We called nests polygyne if they had at least one pairwise relationship ≤0.19, or if the nest did not fit the monogyne criteria. We classified pairwise comparisons between workers as full siblings (≥0.55), related (0.19–0.55) and unrelated (≤0.19).

Fine-scale population structure in *F. paralugubris*

We analyzed the genetic ancestry of the *F. paralugubris* nests collected in 2021 (n = 32) in the population to evaluate potential presence of supercolonies. We created a bed file using the 2021 *F. paralugubris* filtered variants from the species-specific VCF file, excluding chromosome 3, using the flag --make-bed in *PLINK* version 1.90b6.25 (Purcell et al. 2007). We used the bed file as input and calculated genetic ancestry (K = 1–10) for each worker in *ADMIXTURE* version 1.3.0 (Alexander et al. 2009). We identified the number of ancestry groups (K) for the population using cross-validation error scores. We then estimated the nest ancestry by averaging worker ancestry within a nest and mapped the nest according to colony GPS coordinates taken at the time of sampling.

Analysis of the supercolonial species *F. aquilonia* and *F. polyctena*

We examined supergene variation in supercolonial populations of *F. aquilonia*, *F. polyctena*, and their hybrids by leveraging publicly available filtered variants in VCF format from whole-genome resequencing data (Nouhaud et al. 2022b; Portinha et al. 2022; SpecIAnt 2022). Nouhaud et al. aligned their sequence data to the same reference genome (GenBank GCA_907163055.1), so we used the filtered variants to perform subsequent analyses. We first identified the supergene genotypes on chromosome 3 by performing a PCA and calculated heterozygosity on chromosome 3 using *PLINK* version 1.90b6.25 (Purcell et al. 2007) and *VCFtools* version 0.1.17 (Danecek et al. 2011), respectively. We then extracted genotype calls for chromosome 3 using the flag --012 in *VCFtools* version 0.1.17 (Danecek et al. 2011). We calculated the frequency of non-reference alleles averaged across chromosome 3 to confirm the genotypes present in *F. aquilonia*, *F. polyctena*, and *F. aquilonia* × *polyctena* hybrids and omitted missing genotypes as described in the *Supergene genotypes* section.

Software for figures

All plots were produced in R version 4.2.3 (R Core Team 2023) with the package *ggplot2* (Wickham 2016). We mapped nests and their respective intra-nest average ancestry in the program QGIS version 3.30.0 RC (QGIS Development Team 2009). We used the package *cowplot* (Wilke et al. 2024) in R version 4.2.3 to make Figures 2, 3, and 5, and Supplementary Figures S1–S6 and combined the plots in figure panel 4 with Adobe Illustrator. We edited figure legends in Adobe Illustrator. We made Figure 1 in Microsoft PowerPoint.

Results

Absence of *P* haplotype in a *F. paralugubris* population

To identify supergene genotypes in the population, we examined SNPs on chromosome 3 using several analyses. A PCA for each chromosome in *F. paralugubris* (Supplementary Figure S1) showed weak clustering consistent with spatial

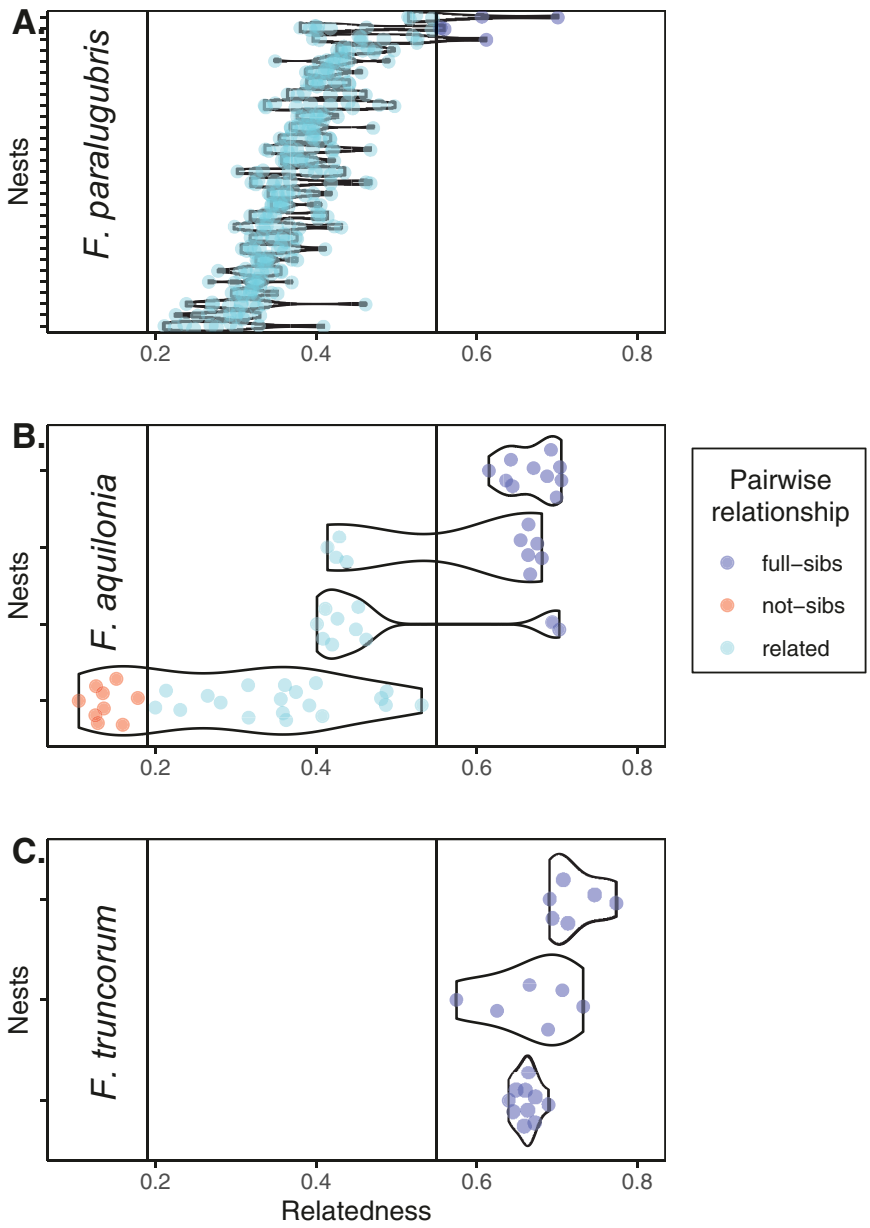

Figure 3. Within-nest relatedness was used to infer whether a nest was monogyne or polygyne. Each point represents a pairwise comparison between workers from the same nest. Points represent full-sibling pairwise relationships (pairwise $R \geq 0.55$), pairs of related individuals (pairwise R between 0.19 and 0.55), and non-sibling pairwise relationships (pairwise $R \leq 0.19$). Vertical lines at $R = 0.19$ and $R = 0.55$ show thresholds between inferred relationship types. Nests with a minimum of four sequenced workers were included in these analyses. (A) In *F. paralugubris* ($n = 29$), we inferred all nests as polygyne. All but three nests lacked full-sibling relationships; the three nests with inferred full-sibling pairs lacked the bimodal distribution of relatedness values expected in monogyne polyandrous colonies. (B) In *F. aquilonia* ($n = 4$), three nests with the *M*/*M* genotype were inferred to be monogyne (two with polyandry), and one colony with *M*/*P* workers was inferred to be polygyne. (C) In *F. truncorum* ($n = 3$), we found all nests to be monogyne with the *M*/*M* genotype.

genetic structure on all chromosomes, including chromosome 3, with no evidence for supergene polymorphism. We then used a PCA with all our species together with two *F. truncorum* individuals with known *M* and *P* genotypes: because of the ancient origin of the queen number supergene in *Formica* (Purcell et al. 2021), we would expect individuals to cluster together on chromosome 3 based on their supergene genotypes instead of species. Along principal component 1, we would expect homozygous individuals for either the *M* or *P* haplotype to group with the respective *F. truncorum* males that served as positive controls. Individuals with the heterozygous *M*/*P* genotype would be expected to show intermediate PC1 values. We identified a uniform cluster for *F. paralugubris* indicating a lack of supergene polymorphism on chromosome 3 (Figure 2B). Two distinct clusters were present for *F. aquilonia* workers. The sampled *F. truncorum* workers formed one uniform cluster. The haploid *M* male grouped with all the sampled *F. truncorum* workers indicating they were *M*-like at the supergene (Figure 2B). The haploid *P* male did not group with any *F. truncorum* indicating we did not have any homozygous *P*-like workers (Figure 2B). The placement of the haploid males indicated that PC1 distinguished the chromosome 3 haplotypes (*M*-like vs *P*-like), while PC2 separated *F. truncorum* with the *M* haplotype from all other

samples. One *F. aquilonia* cluster had PC1 values intermediate between the known *F. truncorum P* and *M* males on PC1 (Figure 2B). All other workers in our analysis were *M*-like homozygotes, which was further supported by heterozygosity estimates (Figures 2B; Supplementary Figure S2A). The *F. aquilonia* cluster intermediate on PC1 and with negative F_{IS} suggested that these individuals were heterozygous at the supergene (Supplementary Figure S2A).

To further examine supergene variation, we calculated the average frequency of non-reference alleles on chromosome 3 (Supplementary Figure S2B). The known *F. truncorum M* and *P* males had an average frequency of non-reference alleles across chromosome 3 of 0.152 and 0.339, respectively. The median across chromosome 3 for all *M*-like homozygous *F. truncorum* workers was 0.172. We found a similar median for all *M*-like homozygous *F. aquilonia* and *F. paralugubris* workers, 0.147 and 0.132, respectively. For the *F. aquilonia* colony with exclusively heterozygous workers, we found the median frequency of non-reference alleles to be 0.255. Through this analysis, we establish that the male sequenced for the reference genome (Nouhaud et al. 2022b) is haploid for the *M* haplotype as it is more genetically similar to the *M*-like workers than the heterozygous workers and known *P* male in our dataset. Hereafter, we will refer to the *M*-like and *P*-like haplotypes as *M* and *P*.

We also examined clustering patterns across the genome using chromosome-level and windowed PCAs to potentially find an association between supercoloniality and other parts of the genome. We didn't find any patterns suggesting other haplotypes were present with chromosome-level PCAs (Supplementary Figures S3–S5) or 750 kb windowed PCAs across the genome (Supplementary Figure S6). However, our reduced representation approach with ddRADs could miss smaller-scale polymorphisms that would otherwise be identified with whole-genome sequencing.

Relatedness and fine-scale population structure support supercoloniality in *F. paralugubris*

We estimated relatedness within each nest to infer nest queen number. We found support for all the *F. paralugubris* workers belonging to polygyne nests with varying degrees of inbreeding (Figure 3A; Supplementary Table S2). A history of inbreeding can increase pairwise relatedness values and complicate the inference of queen number in a nest. In nests belonging to a supercolony with frequent within-nest mating, we would expect moderately high relatedness but not the bimodal distribution that is expected in monogyne polyandrous nests. To further evaluate supercoloniality, we estimated genetic ancestry and admixture in *F. paralugubris*. We found support for three genetic ancestry groups ($K = 3$) in the population (Figure 4; Supplementary Table S3). Each genetic group included multiple nests and occupied a distinct region of our study area (Figure 4A). The spatially distinct genetic groups and consistent polygynous status suggest that this *F. paralugubris* population consists of three supercolonies.

Supergene genotypes and social structure in *F. aquilonia* and *F. truncorum*

In our limited number of *F. aquilonia* nests ($n = 4$), nest average pairwise relatedness between workers ranged between 0.288 and 0.670 (Figure 3B; Supplementary Table S4). The one nest with heterozygous *M/P* workers had the lowest mean relatedness (0.288) and a unimodal distribution of relatedness values (Figure 3B), suggesting that this colony was polygynous. We found the three *M/M* nests had intra-nest pairwise relatedness ranges between 0.401 to 0.703, 0.414 to 0.681, and 0.615 to 0.705. The pairwise relatedness in *M/M* nests suggested monogyne, monandrous social organization in one nest and monogyne, polyandrous in the other two (Figure 3B; Supplementary Table S4). All the *F. truncorum* nests belonged to monogyne, monandrous social nests as pairwise relatedness between *F. truncorum* nestmate workers ranged from 0.575 to 0.774 (Figure 3C; Supplementary Table S5).

The *P* haplotype is absent in supercolonial *F. aquilonia* and *F. aquilonia* × *polyctena* hybrid populations, but present in *F. polyctena*

To assess the generality of our findings in *F. paralugubris*, we reanalyzed published whole-genome sequencing data from other supercolonial species to determine if the *P* haplotype was also absent in *F. aquilonia*, *F. polyctena*, and *F. aquilonia* × *polyctena* hybrids. Of 59 total individuals, only four individuals had heterozygous genotypes on chromosome 3 (Figure 5A). Upon examining supergene variation, we found that the four *F. polyctena* individuals were the only ones with a *P* haplotype, with the remaining 55 individuals being homozygous for the *M* haplotype (39 *F. aquilonia* × *polyctena* hybrids, 10 *F. aquilonia*, and 6 *F. polyctena*, Figure 5A). We further validated the genotypes for each individual by comparison to the *M* haplotype reference genome: the individuals harbouring the *M/P* genotype consistently had a higher frequency of non-reference alleles compared to individuals harbouring the *M/M* genotype (Figure 5B). These results demonstrate that the *P* haplotype is not required for supercoloniality in other *F. rufa* group species, but additional nest-level analyses are warranted to assess the effect of the *P* haplotype in *F. polyctena*.

Discussion

Contrary to our initial expectations, we found that the supergene haplotype associated with polygyny across the *Formica* genus is absent or rare in highly polygynous supercolonial populations of multiple species. In one population of *F. paralugubris*, nests were polygynous despite workers exclusively having the *M/M* genotype (Figures 2B and 3A). This is an unexpected deviation; in other *Formica* species, colonies composed entirely of *M/M* genotypes are almost always monogynous (e.g., Brelsford et al. 2020; Lagunas-Robles et al. 2021; McGuire et al. 2022; Pierce et al. 2022; Purcell et al. 2014; Scarparo et al. 2023). Secondly, as expected, we find signatures of the supercolonial social organization in the population of *F. paralugubris* (Figures 3A and 4). The average intra-nest ancestry suggests the presence of three supercolonies with spatial boundaries. The spatial scale of these supercolonies is consistent with studies in other populations of *F. paralugubris* (Holzer et al. 2009). Lastly, we show a lack of *P* haplotypes in additional supercolonial *Formica rufa* group species. We found this in *F. aquilonia* and *F. aquilonia* × *polyctena* hybrids, but not *F. polyctena* (Figure 5). Interestingly, in our sampling population, we observed a polygyne *F. aquilonia* nest with heterozygous *M/P* individuals (Figures 2B and 3B). This suggests that the *P* haplotype may be present in some supercolonial species, but that it is not necessary for supercolonial social organization.

Did supercoloniality originate from polygyny?

Supercoloniality is generally thought to be an extension of polygyny (Helanterä 2022; Helanterä et al. 2009). For example, in the common red ant *Myrmica rubra*, there are no differences in body size between individuals from polygynous and supercolony nests suggesting that morphological changes are not needed for supercoloniality (Huszár et al. 2014). However, morphological differences are observed when compared to individuals from monogyne nests. Additionally, in the Argentine ant *Linepithema humile*, differences in colony size are likely associated with local ecological conditions and not any life history changes (Pedersen et al. 2006), suggesting that native polydomous polygyne colonies (Suarez et al. 2008) expand into supercolonies given favourable environments. In contrast to these examples outside the *Formica* genus, our finding that the supergene haplotype associated with polygyny across *Formica* is absent in supercolonial populations of multiple species raises the possibility that supercoloniality can be a qualitatively different type of polygyny with a distinct origin and distinct genetic mechanism.

Some *Formica* species exhibit monogyne, polygyne, and supercolonial social strategies. In two of these species, *Formica truncorum* and *Formica exsecta*, the presence or absence of the *P* haplotype is associated with social

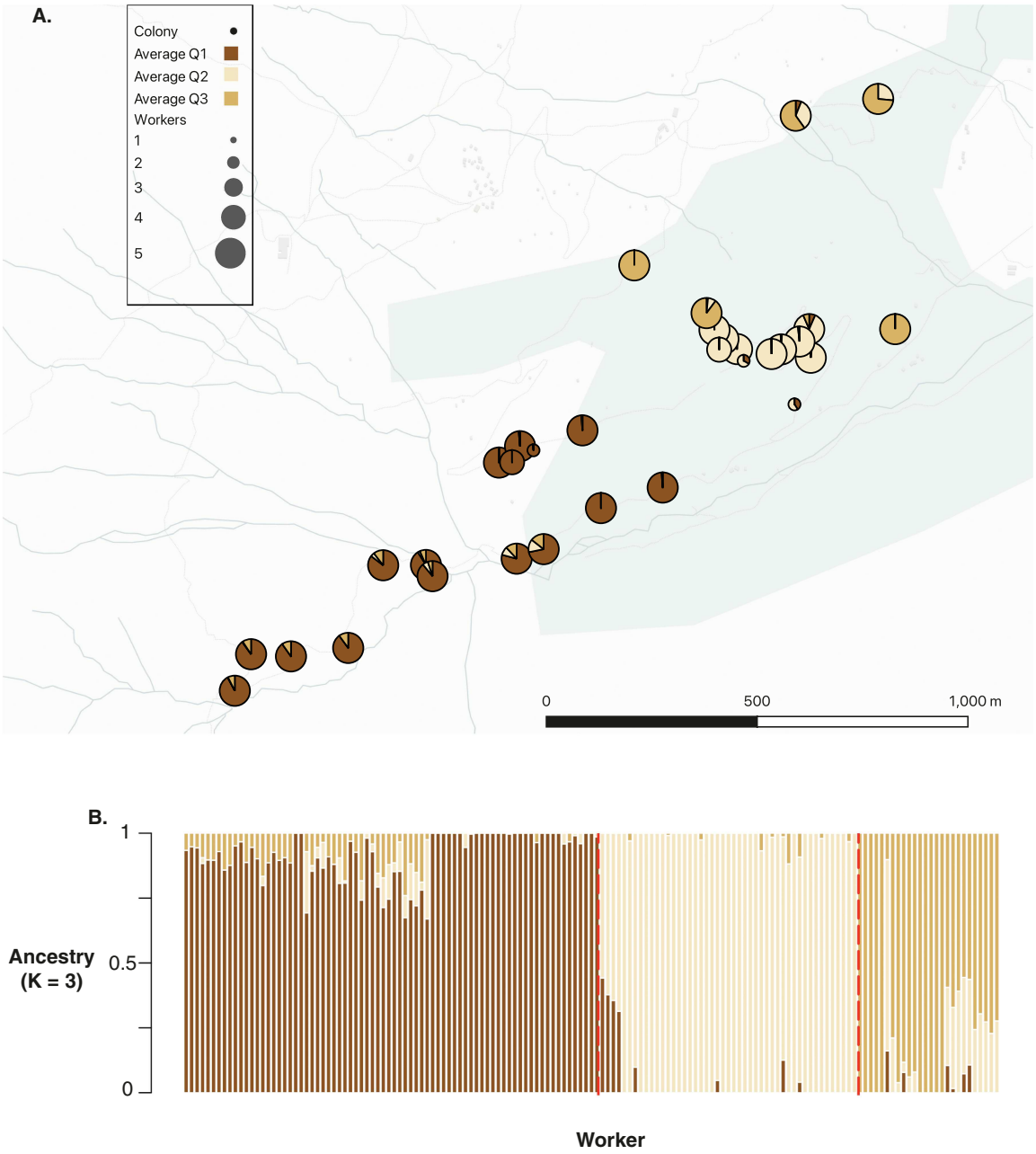

Figure 4. Admixture results suggest the presence of three *F. paralugubris* genetic ancestry groups. Colours represent the proportion of ancestry for each nest or individual. (A) Each nest was plotted based on its physical location. Pie charts represent the average nest ancestry. We found three genetic groups that are spatially separated. (B) ADMIXTURE results for worker ancestry ($K = 3$). Individual worker ancestries are represented by each bar. The bars are ordered by increasing latitude. The boundaries between the three supercolonies are delineated by dashed vertical bars.

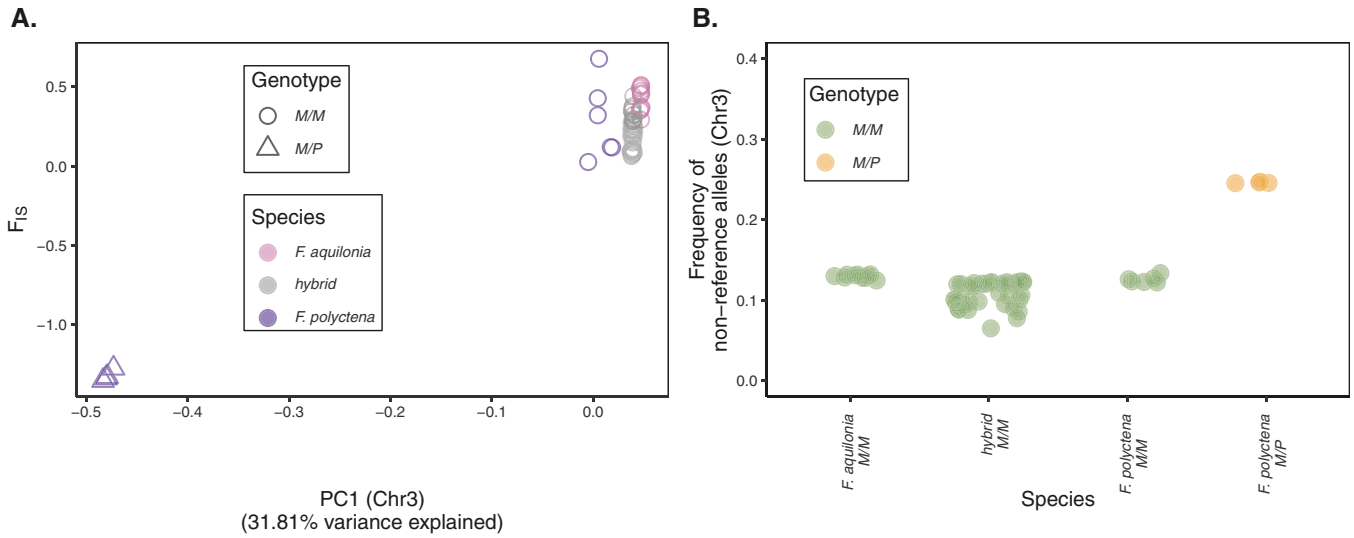

Figure 5. Additional species in the *F. rufa* group, *F. aquilonia* ($n = 10$), *F. polyctena* ($n = 10$), and *F. aquilonia* × *polyctena* hybrids ($n = 39$), from hybridizing supercolonial populations show that the *P* haplotype is rare in supercolonial populations. Each point represents an individual. (A) Chromosome 3 PC1 plotted against chromosome 3 F_{IS} supported the genotype assignments as positive F_{IS} values suggest the individual is homozygous on chromosome 3, while negative F_{IS} values suggest the individual is heterozygous on chromosome 3. All *F. aquilonia* ($n = 10$) and *F. aquilonia* × *polyctena* hybrids ($n = 39$) were *M*/*M* at the supergene, while four of 10 *F. polyctena* were *M*/*P* at the supergene. (B) The frequency of non-reference alleles on chromosome 3 shows that four of 10 *F. polyctena* workers are substantially more divergent from the *M* reference genome than the rest of the workers in this dataset, suggesting that these four workers carry at least one copy of the *P* haplotype.

organization in non-supercolonial populations (Brelsford et al. 2020). The same may be true of *F. aquilonia* (Figure 3B). These species present an excellent opportunity to identify the genetic basis of supercoloniality: a genome-wide association study for social organization in species exhibiting all three strategies could uncover variants that are specific to supercolonies, whether on the *M* haplotype or on other chromosomes. A recent study showed that two duplicated *P*-specific paralogous genes were found on an *M* haplotype background in *F. paralugubris* and *F. aquilonia*, but supercolonial *F. polyctena* still had the *P* haplotype (Sigeman et al. 2024). These paralogs may be promising causal loci. Identifying causal loci would provide insight into whether this life history strategy emerged once or multiple times in this set of species. In the case of a single origin in the *F. rufa* group, identifying the causal locus would also determine whether supercoloniality emerged in the common ancestor of the species group, or emerged more recently and introgressed across species boundaries.

What is the role of the *P* haplotype in supercolonial *Formica* ants?

If the *P* haplotype is not necessary for supercoloniality, what role does the *P* haplotype play in supercolonial *Formica* species? One possibility is that some populations of facultatively supercolonial species retain the ancestral supergene-determined social polymorphism, with single-nest colonies headed by either a single queen or multiple queens depending on the presence or absence of the *P* haplotype. Another possibility, not mutually exclusive, is that the *P* haplotype is retained in supercolonial populations due to its effects on traits other than queen number. Morphological, behavioural, and population genetic evidence suggests that dispersal is typically lower for individuals from polygyne vs monogyne colonies (De Gasperin et al. 2024; Hakala et al. 2019; Keller 1993, 1995; Sundström et al. 2005). If supercolonial species are subject to frequency-dependent or spatially variable selection on dispersal, this could account for the maintenance of supergene polymorphism in the absence of queen number polymorphism.

Conclusion

We find that supercoloniality is not determined by the *P* haplotype. Despite *F. paralugubris* only having polygyne nests, all the workers were *M*/*M* at the queen number supergene. The striking deviation in *F. paralugubris* between a queen number supergene haplotype and nest phenotype contrasts with the long-standing association between the *P* haplotype and polygyny observed in previous studies of non-supercolonial *Formica* species. We observed a similar discordance in supercolonial *F. aquilonia* and *F. aquilonia* × *polyctena* hybrids, where all individuals lacked the *P* haplotype. Additionally, supercolonial *F. polyctena* had the *P* haplotype despite its absence in *F. aquilonia* × *polyctena* hybrids. Our results show that the presence of the *P* haplotype is not necessary for supercolonial organization in some species in the *F. rufa* group, but that is it nevertheless retained in socially polymorphic populations of the same species. This suggests that the evolutionary origin and genetic mechanism of supercoloniality may be qualitatively distinct from the origin and mechanism of polygyny found in single-nest colonies.

Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

Data availability

Worker and corresponding nest metadata and relatedness estimates are included in the supplementary material. Variant Call Format files and data files and scripts

necessary to reproduce the figures were deposited on Dryad (DOI 10.5061/dryad.4qrfj6qmm). Raw sequence reads were deposited on the Sequence Read Archive of the National Center for Biotechnology Information (Bioproject PRJNA1200681).

Author contributions

German Lagunas-Robles (Conceptualization [supporting], Formal analysis [lead], Funding acquisition [supporting], Investigation [equal], Supervision [equal], Visualization [lead], Writing—original draft [lead], Writing—review & editing [equal]), Zul Alam (Formal analysis [supporting], Investigation [equal], Writing—review & editing [supporting]), and Alan Brelsford (Conceptualization [lead], Funding acquisition [lead], Investigation [equal], Resources [lead], Supervision [equal], Visualization [supporting], Writing—review & editing [equal])

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Conflicts of interest

None declared.

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