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## The maintenance of polymorphism in an ancient social supergene

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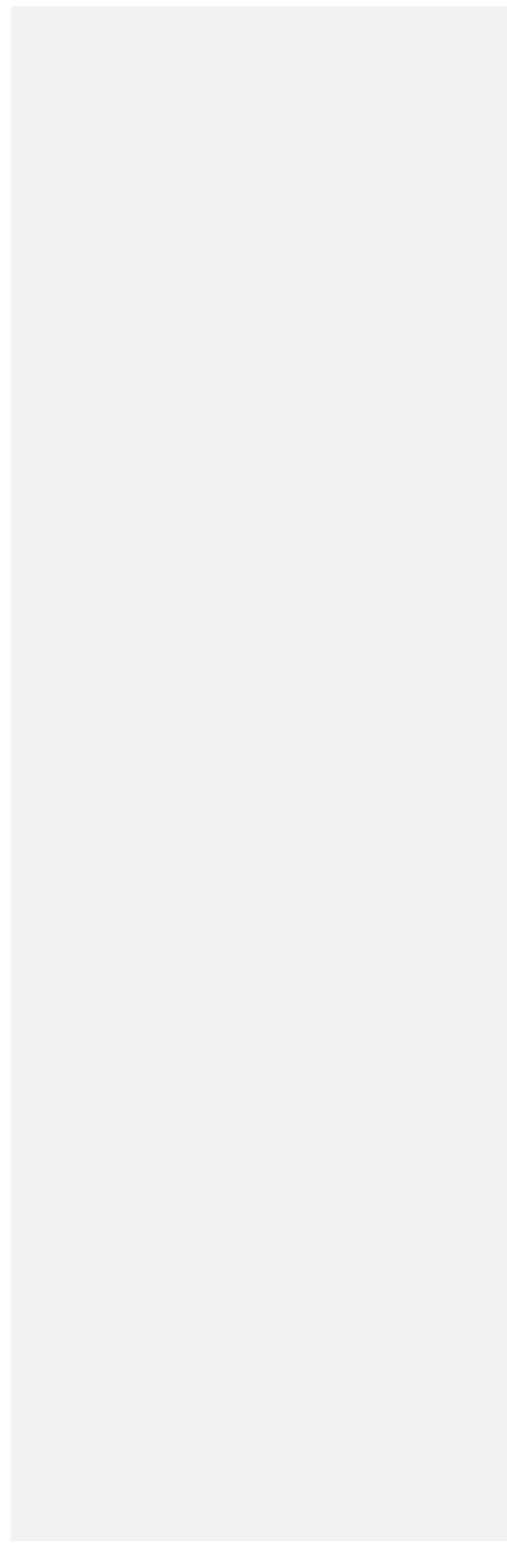
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**Running title:** Polymorphism in an ancient supergene



30 **Abstract**

31  
32 Supergenes, regions of the genome with suppressed recombination between sets of  
33 functional mutations, contribute to the evolution of complex phenotypes in diverse systems.  
34 Excluding sex chromosomes, most supergenes discovered so far appear to be young, being found  
35 in one species or a few closely related species. Here, we investigate how a chromosome  
36 harboring an ancient supergene has evolved over about 30 Ma. The *Formica* supergene  
37 underlies variation in colony queen number in at least five species. We expand previous  
38 analyses of sequence divergence on this chromosome to encompass about 90 species spanning  
39 the *Formica* phylogeny. Within the non-recombining region, the gene *knockout* contains 22  
40 single nucleotide polymorphisms (SNPs) that are consistently differentiated between two  
41 alternative supergene haplotypes in divergent European *Formica* species, and we show that these  
42 same SNPs are present in most *Formica* clades. In these clades, including an early diverging  
43 Nearctic *Formica* clade, individuals with alternative genotypes at *knockout* also have higher  
44 differentiation in other portions of this chromosome. We identify hotspots of SNPs along this  
45 chromosome that are present in multiple *Formica* clades to detect genes that may have  
46 contributed to the emergence and maintenance of the genetic polymorphism. Finally, we infer  
47 the presence of three gene duplications on one haplotype, based on apparent heterozygosity  
48 within these genes in the genomes of haploid males. This study strengthens the evidence that this  
49 supergene originated early in the evolution of *Formica* and that just a few loci in this large  
50 region of suppressed recombination retain strongly differentiated alleles across contemporary  
51 *Formica* lineages.

52 **Keywords:** Formicinae, coadapted gene complex, suppressed recombination, gene flux

## Introduction

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Autosomal supergenes, sets of functional mutations linked in a region of suppressed or greatly reduced recombination, are associated with a variety of complex phenotypes in diverse eukaryotic lineages (Charlesworth 2016; Schwander et al. 2014; Thompson & Jiggins 2014). Supergenes underlie phenotypic polymorphisms including mating systems in fungi (Branco et al. 2018; Sun et al. 2019), reproductive strategies in plants (e.g. Huu et al. 2020, Li et al. 2016) and birds (e.g. Küpper et al. 2016; Lamichhaney et al. 2016; Tuttle et al. 2016), mimetic wing coloration in butterflies (Joron et al. 2011), and social organization in ants (Purcell et al. 2014; Wang et al. 2013). Known supergenes differ substantially in their age, with some present in a single species (e.g. ruffs, Küpper et al. 2016, Lamichhaney et al. 2016) and some spanning a few closely related species (e.g. in fire ants, Yan et al. 2020, Cohen & Privman 2020 and in *Heliconius* butterflies, Kronforst & Papa 2015). Just two autosomal supergenes so far may have an ancient origin: Ramanaukas & Igić (2017, 2021) suggested that the S-RNase-based self-incompatibility mechanism found in many flowering plants may have originated in the common ancestor of the core eudicots, and Brelsford et al. (2020) showed that the *Formica* ant social supergene, which underlies colony queen number and therefore controls polymorphic social organization, has persisted in species spanning an estimated 20-40 Ma of independent evolution.

Ancient supergenes provide an opportunity to investigate the evolutionary trajectory of a region of suppressed recombination across diverging species with different lifestyles. Brelsford et al. (2020) demonstrated that a large region of reduced recombination spans ~10.5 Mbp of a 14 Mbp chromosome in species from three distinct *Formica* clades. Within each of the well-studied species, numerous variants distinguish the two alternative supergene haplotypes (Brelsford et al. 2020). Surprisingly, only 142 single nucleotide polymorphisms (SNPs) were consistently

79 associated with alternative supergene haplotypes across these five species. The 142 trans-species  
80 SNPs were distributed across the supergene region but no SNPs matching this pattern were found  
81 elsewhere in the genome. These SNPs likely provide information about functionally important  
82 regions of the supergene, because they would either be maintained in diverging lineages by  
83 selection or rearrangements preventing recombination between alternative haplotypes. The  
84 discovery of hotspots containing numerous trans-species, haplotype-specific SNPs that are  
85 distributed across the supergene region is consistent with an analytical model investigating the  
86 evolutionary fate of inversions with rare recombination or gene conversion events (Guerrero et  
87 al. 2012). In this 'eroded strata' model (Brelsford et al. 2020), rare lineage-specific recombination  
88 or gene conversion events would have homogenized alternative supergene haplotypes in  
89 different lineages, but selection would have prevented the erosion of SNPs with functional  
90 importance in each of the alternative haplotypes (Table 1 lays out the expectations of this model  
91 at three timescales). In this study, we refer to variants that are found across divergent species as  
92 trans-species, haplotype-specific SNPs.

93 *Formica* species frequently exhibit a polymorphism in colony queen number (e.g.  
94 Chapuisat et al. 2004; DeHeer & Herbers 2004; Seppä et al. 2004; Bargum et al. 2007), with  
95 some colonies having a single queen (= monogyne) and others containing multiple, often  
96 unrelated, queens (= polygyne). These alternative social forms generally conform to the  
97 “polygyny syndrome” where workers and queens are generally smaller, but colony size is larger,  
98 in polygyne compared to monogyne colonies of the same species (Keller 1993; Rosset &  
99 Chapuisat 2007). The discovery of a supergene associated with this variation has helped to shed  
100 light on the proximate forces that maintain this phenotypic polymorphism (Purcell et al. 2014;  
101 Avril et al. 2019, 2020). In *Formica selysi*, queens and workers from monogyne colonies

102 invariably have the Sm/Sm genotype, while individuals from polygyne colonies always have at  
103 least one copy of the Sp haplotype (diploids are either Sm/Sp or Sp/Sp; Purcell et al. 2014).  
104 Through high density linkage mapping, we have shown that recombination occurs across  
105 chromosome 3 in both homozygous forms, but recombination is suppressed in heterozygotes and  
106 there are a series of structural rearrangements between the two haplotypes (Purcell et al. 2014;  
107 Brelsford et al. 2020). More recently, a second linked supergene that is associated with split sex  
108 ratio phenotypes (i.e. whether colonies specialize in producing males or gynes) was identified in  
109 the North American species *Formica glacialis* and *Formica podzolica* (Lagunas-Robles et al.  
110 2021).

111 In this study, we provide a comprehensive look at the evolutionary history of the  
112 chromosome carrying the *Formica* supergene in a comparison of over 90 species spanning the  
113 *Formica* tree of life. The dataset presented by Brelsford et al. (2020) did not include an early-  
114 diverging clade sister to most other *Formica* species (Romiguier et al. 2018, Borowiec et al.  
115 2021), and included only European species. While *Formica* has received less research attention  
116 in the western hemisphere, North America is a hotspot of diversity in this genus. Some speciose  
117 North American clades are species-poor or absent in Europe (Borowiec et al. 2021). Our sample  
118 includes 11 genomes from the *neogagates-pallidefulva* clade, an early-diverging clade that is  
119 exclusively distributed in the New World, as well as several outgroup species, allowing us to  
120 examine the extent of trans-species variation on this chromosome across the entire genus. For 1-  
121 3 individuals per species, we assess whole genome sequence data including 90 new whole  
122 genome sequences that were generated for this study. Whole genome data is necessary, because  
123 we know that the clusters of trans-species, haplotype-specific SNPs are relatively small and  
124 likely to be missed with reduced representation sequencing. In this assessment, we maximize our

125 coverage of species and clades of *Formica*, but we do not yet know whether all of the species  
126 included in our analysis exhibit a polymorphism in colony queen number or sex ratio. We  
127 address the following questions: 1) Brelford et al. (2020) found that the gene *knockout* harbored  
128 the only cluster of SNPs that were consistently found in 15 European species. We ask whether  
129 *knockout* remains a hotspot of trans-species, haplotype-specific SNPs across the whole genus. 2)  
130 Are there sets of divergent SNPs along chromosome 3 in members of the *neogagates-*  
131 *pallidefulva* clade, which is sister to all other *Formica* species in our analysis? If so, this would  
132 suggest that these genomic variants evolved prior to the diversification of the genus *Formica*. 3)  
133 Using our comparative approach, can we identify additional candidate genes that are consistently  
134 associated with alternative *knockout* variants in multiple *Formica* clades? Such genes may have  
135 contributed to selection for suppressed recombination to preserve favorable combinations of  
136 alleles in two or more genes and may continue to contribute to contemporary phenotypic  
137 polymorphisms. Genomic regions containing candidate genes would be characterized by trans-  
138 species, haplotype-specific SNPs in most *Formica* clades. These analyses expand our  
139 understanding of the evolutionary trajectory of the chromosome that harbors the *Formica*  
140 supergene and identify additional candidate genes therein.

141

## 142 **Methods**

143

### 144 *Overview of samples included in the analysis*

145 We obtained sequences from 114 *Formica* samples (1-3 individuals per species, most  
146 samples identified to species by Borowiec et al. 2021) and three outgroup samples (one  
147 *Iberoformica* and two *Polyergus*; Tables 2 and S1). Many samples (n = 80) were collected for a

148 concurrent study investigating the phylogenetic history of the genus *Formica* (Borowiec et al.  
149 2021), 21 were previously assessed by Brelsford et al. (2020), and the remaining 16 were  
150 collected by Purcell and Brelsford (Table S1). We removed six samples from further  
151 consideration due to low sequence coverage and high levels of missing data (Table S1).  
152 Represented species spanned the phylogeny of the genus, including 10 species (11 individuals)  
153 from the *neogagates-pallidefulva* clade, as well as species from seven additional clades endemic  
154 to North America, including the *difficilis* and *integra* clades (Figure 1). Previous karyotyping of  
155 *Formica* species indicates that haploid chromosome number is generally 27 and 26 for non-  
156 parasitic and parasitic species, respectively (Hung 1969, Hauschteck-Jungen & Jungen 1976,  
157 Rosengren et al. 1980). The samples analyzed herein focused on maximizing the number of taxa  
158 represented, so the social structure of the source colony was not assessed, and the genotype of  
159 each individual at the supergene was not predicted in advance. As a result, we focus on assessing  
160 the sequence variation of these samples, and we interpret the supergene status with caution.

161

#### 162 *Whole genome sequencing*

163 For 96 new samples, we extracted genomic DNA using Qiagen DNEasy Blood and  
164 Tissue kits. For 81 samples, we constructed whole genome libraries using KAPA library  
165 preparation kits (n=80) or a Nextera-based library protocol (Henderson and Brelsford 2021;  
166 n=1). These samples were first assigned to species based on morphological assessment (Table  
167 S1). Fifteen additional samples were sent to the UC Berkeley Vincent Coates Genome  
168 Sequencing Laboratory for library preparation (Tables 2, S1). All sequencing was performed by  
169 the UC Berkeley sequencing core, using the HiSeq 4000 platform with 150 bp paired-end reads.

170

171 *Bioinformatics*

172 We merged overlapping pair-end reads using PEAR (v0.9.10; Zhang et al. 2014). We  
173 aligned the reads to the published *F. selysi* chromosome-level genome assembly, which is based  
174 on individuals of monogyne origin (Sm haplotype; Brelsford et al. 2020) using BWA-MEM  
175 (v0.7.17; Li 2013) and removed PCR duplicates using Samtools (v1.8, Li et al. 2009). We called  
176 variants with Samtools mpileup, using only reads with a mapping quality of at least 20. We  
177 filtered the resulting variants using VCFtools (Danecek et al. 2011), retaining genotypes with  
178 minimum depth 2 and loci with missing data in < 50% of individuals. To identify groups of  
179 closely related species within our dataset, we generated a distance matrix based on genome-wide  
180 variants excluding chromosome 3 using Plink (v1.90b3.38, Purcell et al. 2007), and constructed a  
181 neighbor-joining tree using T-REX (Boc et al. 2012).

182

183 *Assessing genotype at knockout*

184 The gene *knockout* contained 22 trans-species, haplotype-specific SNPs that were  
185 consistently associated with queen number variation in five European *Formica* species  
186 (Brelsford et al. 2020). We examined the SNPs in this candidate gene to initially assess genotype  
187 variation in the newly sequenced species. We used VCFtools to export the genotypes of these 22  
188 SNPs in all individuals and observed that most were either homozygous for the reference allele  
189 or were heterozygous.

190

191 *Identifying additional trans-species, haplotype-specific SNPs*

192 We then identified trans-species, haplotype-specific SNPs between the *knockout*  
193 homozygotes and heterozygotes of six clades of *Formica* along chromosome 3. In addition to



194 focusing on the early diverging *neogagates-pallidefulva* clade, we examined five other clades  
195 that included at least four individuals with each of the alternative multi-locus genotypes at  
196 *knockout*, including *fusca* #3, *fusca* #4, and *fusca* #5 clades, as well as two larger clades, one  
197 containing all socially parasitic species and the other containing *fusca* clades #3, #4, and #5. We  
198 used the --hardy function of VCFtools to count the number of individuals homozygous for the  
199 reference allele, homozygous for the alternate allele, and heterozygous at each locus, separately  
200 for *knockout* homozygotes and heterozygotes. We then used a custom script to identify SNPs that  
201 were heterozygous in all genotyped *knockout* heterozygotes, and homozygous for the same allele  
202 (either reference or alternate) in all genotyped *knockout* homozygotes, retaining only SNPs that  
203 were genotyped in at least three heterozygous and three homozygous individuals in the focal  
204 clade. In the *neogagates-pallidefulva* clade, we searched for these SNPs genome-wide. Since the  
205 majority were found on chromosome 3, we plotted the number of SNPs meeting these criteria in  
206 1 kbp windows along chromosome 3 for all comparisons. We identified positions along the  
207 chromosome that consistently contained multiple trans-species, haplotype-specific SNPs in most  
208 or all of the six clades. These hotspots of trans-species differentiation are more likely to harbor  
209 genetic variants important in the origin and persistence of the supergene polymorphism. In  
210 addition, we compared the trans-species, haplotype-specific SNPs identified in the *neogagates-*  
211 *pallidefulva* clade across *fusca* clade #3, *exsecta* clade, and *rufa* clade, which were previously  
212 examined by Brelsford et al. (2020) and contain samples of known social origin and supergene  
213 status.

214

215 *Genes overlapping hotspots of differentiation between alternative haplotypes*

216 For four regions with high concentrations of trans-species, haplotype-specific SNPs, we  
217 used the annotated genome of *F. exsecta* (Dhaygude et al. 2019) to identify overlapping genes.  
218 We extracted the *F. selysi* reference genome sequence for each of these regions and aligned these  
219 sequences to the *F. exsecta* genome using BLAST (Altschul et al. 1997). We then scanned the *F.*  
220 *exsecta* annotation for genes overlapping these regions, downloaded the coding sequences of  
221 these genes, and aligned them back to the *F. selysi* reference to obtain the approximate locations  
222 of exons in *F. selysi*.

223

224

## Results

225 *Are knockout SNPs polymorphic within other Formica clades?*

226 Variants spanning 8-22 SNPs in the gene *knockout* were detected in 11 out of 14 *Formica*  
227 clades examined in this dataset, including the *neogagates-pallidefulva* clade (Fig. 1). This  
228 comparison included 8 clades that were not represented in a previous trans-species analysis of  
229 the *Formica* supergene (Brelsford et al. 2020). In most of these new clades (6 out of 8), we  
230 detected individuals that were either homozygous for the *knockout* reference allele (based on  
231 alignment to the *F. selysi* reference genome, which is assembled from an individual of monogyne  
232 origin with the 'Sm' haplotype) or heterozygous. In the remaining two new clades, the *difficilis*  
233 and *dakotensis* clades, we detected only individuals homozygous at the reference allele (Fig. 1).

234 Since the SNPs distinguishing these groups are shared across divergent species, we infer  
235 that these genotypes are homologous to those previously identified in individuals of monogyne  
236 or polygyne origin (Brelsford et al. 2020, Fig. 1), although we cannot link alternative genotypes  
237 to phenotypes for most species in this dataset. For consistency within this study and across  
238 related studies, we refer to the three *knockout* multi-locus genotypes as 'Sm/Sm' (homozygous,

239 alleles match reference genome), 'Sm/Sp' (heterozygous), or 'Sp/Sp' (homozygous for the  
240 alternative allele). Notably, we detected very few individuals that appeared Sp/Sp at *knockout*  
241 (again, we cannot infer an association with polygyny based on current data). Overall, we  
242 identified five Sp/Sp workers, plus three Sp males. All but one of these individuals were  
243 selected for sequencing to represent the Sp haplotype in a previous study (Brelsford et al. 2020).  
244 In the present study, we successfully sequenced 86 new *Formica* workers and two new males  
245 without prior knowledge of their colony social structure. Of these, 63 workers were Sm/Sm at  
246 *knockout*, 22 were Sm/Sp, and one was putatively Sp/Sp (Table S1, Fig. 1). Both males had the  
247 Sm haplotype at *knockout*.

248 *Were haplotype-specific SNPs present in the common ancestor of extant Formica?*

249       Based on their genotypes at the gene *knockout*, our sample of *neogagates-pallidefulva*  
250 clade members included five Sm/Sp heterozygotes and six Sm/Sm homozygotes. Since this  
251 Nearctic clade diverged from other *Formica* clades very early, approximately 26 Ma ago, we  
252 inferred that haplotype-specific SNPs were present in the common ancestor of the genus  
253 *Formica*. Although we only sequenced a single *Iberoformica subrufa* individual, 19 of the 20  
254 aligned *knockout* SNPs also matched the reference allele, suggesting that the reference haplotype  
255 was already present in the most recent common ancestor of *Formica* and *Iberoformica*. Both  
256 *Polyergus* samples exhibited a mix of reference and alternative alleles across this region.

257       Interestingly, some individuals in the *neogagates-pallidefulva* clade may carry a third  
258 haplotype at the *knockout* locus. These individuals exhibited the Sm/Sm genotype at positions  
259 11,910,116-11,910,323 bp and the Sm/Sp genotype at positions 11,910,356-11,910,880 bp  
260 within our panel of 22 SNPs (Fig. 1). We considered individuals with a majority of Sm/Sp  
261 genotypes from 11,910,356-11,910,880 bp, including both *F. incerta* individuals, *F. pallidefulva*,

262 *F. lasioides*, and *F. obtusopilosa*, to be heterozygous for subsequent analyses. By comparing the  
263 *knockout* heterozygotes and homozygotes from this clade, we identified trans-species, haplotype-  
264 specific SNPs specific to the *neogagates-pallidefulva* clade. This analysis yielded a total of 429  
265 SNPs on chromosome 3. We found an additional 393 trans-species SNPs distributed in the rest of  
266 the genome, with the number of such SNPs per chromosome ranging from 1 on chromosome 18  
267 to 69 on chromosome 17. With the exception of 2 SNPs that were found in the region from 0-2  
268 Mbp, the SNPs on chromosome 3 spanned the 10.5 Mbp region that harbors the social supergene  
269 in other *Formica* species (Fig. 2, Purcell et al. 2014; Brelsford et al. 2020). In addition to the  
270 peak of differentiation that overlaps with *knockout* (at 11.9 Mbp), there are notable peaks  
271 harboring large numbers of SNPs at 2.3, 11.6, and 12.4 Mbp (Fig. 2A).

272 We assessed the number and position of trans-species, haplotype-specific SNPs along  
273 chromosome 3 within members of the *neogagates-pallidefulva* clade (Fig. 2A), within the  
274 socially parasitic *Formica* species, including the *exsecta*, *sanguinea*, *dakotensis*, *rufa*, *integra*,  
275 and *difficilis* clades (Fig. 2B), within the well-sampled *fusca* clades #3, 4, and 5 (Fig. 2C), and  
276 within each of these three *fusca* clades, #3 (Fig. 2D), #4 (Fig. 2E), and #5 (Fig. 2F). Across  
277 these comparisons, several small areas harboring numerous trans-species, haplotype-specific  
278 SNPs were consistently found at 2.3, 11.6, and 11.9 Mbp (the latter includes *knockout*, which  
279 was used to diagnose haplotype; Figs. 1, 2). We also observe peaks of differentiation at 12.4  
280 Mbp in the *neogagates-pallidefulva* clade and in *fusca* clade #3.

281

282 *Were additional candidate genes associated with alternative knockout variants in multiple*  
283 *clades?*

284 We assessed the genes in the neighborhood of trans-species, haplotype-specific SNPs  
285 detected in the *neogagates-pallidefulva* clade. In addition to the gene *knockout*, we identified six  
286 other genes localized at or near one of four peaks of divergences between putative Sm/Sm and  
287 Sm/Sp individuals in this clade (Fig. 2A, Fig. 3). Like *knockout*, several of these additional  
288 genes are involved in neural development in *Drosophila* (Table 3) and may have contributed to  
289 the origin of the supergene.

290 To determine whether alternative alleles at these genes are likely to be maintained by  
291 selection in other *Formica* species, we then investigated the *neogagates-pallidefulva* clade trans-  
292 species, haplotype-specific SNPs in the workers and males from *exsecta*, *rufa*, and *fusca* clade #3  
293 that include individuals with known monogyne or polygyne backgrounds, as described by  
294 Brelsford et al. (2020; see Table S1 and Fig. 1). For two of the four peaks of differentiation, at  
295 2.3 Mbp and 12.4 Mbp along chromosome 3, we found that the haploid Sp males (and Sp/Sp  
296 homozygotes) appeared to be heterozygous (Fig. 3). This finding suggests a possible gene  
297 duplication event in the Sp haplotype: haploid individuals cannot be heterozygous, so the  
298 apparent heterozygosity we observe in Sp males must result from these individuals carrying two  
299 divergent copies of these regions of the genome. At an additional peak of differentiation at 11.6  
300 Mbp within the supergene, we observed that only the *fusca* clade #3 Sp male and Sp/Sp workers  
301 appeared to be heterozygous, suggesting a possible lineage-specific duplication. Intriguingly, all  
302 of these clusters of differentiation overlap with annotated genes (Table 3). In the case of *single-*  
303  *minded*, all of the variants that differ between the two putative copies of the gene are found in  
304 introns (Fig. 3B). In contrast, the variants we detected in *AmGR10* include 100 SNPs in the  
305 introns and 32 in exons (Fig. 3C).

306

307

## Discussion

308 By screening 111 whole genome sequences spanning the *Formica* phylogeny, we provide  
309 strong evidence that trans-species, haplotype-specific SNPs in the gene *knockout* that were  
310 originally identified and associated with colony social structure in five well-studied species  
311 (Brelsford et al. 2020) can be traced back to at least the common ancestor of extant *Formica*  
312 species (Fig. 1). By comparing differences between the genomes of individuals that are  
313 heterozygous and homozygous at *knockout* with a focus on the previously unsampled  
314 *neogagates-pallidefulva* clade (Borowiec et al. 2021), we discover additional clusters of trans-  
315 species, haplotype-specific SNPs that align to other regions of chromosome 3. This suggests first  
316 that all or part of the two *F. selysi* supergene variants formed in the common ancestor of all  
317 extant *Formica*. Second, these regions harbor additional genes that are candidates for  
318 contributing to the formation of the supergene and, perhaps, the contemporary phenotypes  
319 associated with it.

320 What conditions could lead to the maintenance of ancient trans-species, haplotype-  
321 specific SNPs distributed in several hotspots along chromosome 3 for approximately 30 Ma,  
322 through numerous speciation events? The patterns identified in this comparison are consistent  
323 with the predictions of the eroded strata model (predictions and empirical support are described  
324 in Table 1), but more information is needed to determine whether one or more inversions were  
325 already present in the common ancestor of contemporary *Formica* species. We emphasize the  
326 importance of investigating synteny in the genus to reconstruct the history of structural  
327 rearrangements between and within the Sp and Sm haplotype groups, because the peaks of  
328 differentiation spaced far apart on the *F. selysi* Sm reference genome could be in tight physical  
329 linkage on the Sp or Sm haplotypes in other *Formica* clades. The two largest clusters of trans-

330 species, haplotype-specific SNPs in the *neogagates-pallidefulva* clade occur near the ends of the  
331 *F. selysi* supergene, assuming that gene order is conserved between the *F. selysi* reference  
332 genome and members of this clade (Fig. 2). These could reflect the accumulation of mutations  
333 that are protected from rare recombination events due to their proximity to inversion breakpoints,  
334 particularly if the supergene initiated through one large inversion. However, we also find  
335 preliminary evidence that these regions have been duplicated in the putative Sp haplotype in  
336 several clades. In the haploid Sp males and Sp/Sp workers of *F. selysi*, *F. cinerea*, *F. lemani*, *F.*  
337 *exsecta*, and *F. truncorum*, many SNPs appear to be heterozygous in these two regions (Fig. 3).  
338 In the haploid Sp males especially, stretches of apparent heterozygosity likely reflect gene  
339 duplications. Here, Sp males appear to have two copies of each of these regions, one similar to  
340 the version present on the Sm haplotype and another that is substantially divergent. Gene  
341 duplications can accumulate in regions of suppressed recombination (e.g. Huu et al. 2020), as  
342 observed in the *S. invicta* supergene (Fontana et al. 2020). Future work on gene expression may  
343 reveal whether duplicated copies of these genes result in neofunctionalization,  
344 subfunctionalization or the formation of pseudogenes (e.g. Dang et al. 2019).

345 Through these analyses of additional *Formica* species, we have identified new genes that  
346 are highly differentiated between individuals that are Sm/Sm and Sm/Sp at *knockout*. These  
347 genes were not detected in the initial trans-species comparison reported by Brelsford et al.  
348 (2020). Two of these genes occur within the putative duplicated regions (*single-minded* and  
349 *FMRFaR*), while three more genes are found in the differentiated region upstream of *knockout*  
350 that appears to be duplicated on the Sp haplotype of *fusca* clade #3 (*AMGR10*, *tplus3b*, and an  
351 uncharacterized protein that may be a transcription factor). Like *knockout*, *single-minded* is  
352 associated with the development of the nervous system in *D. melanogaster* (Umetsu et al. 2006).

353 *FMRFaR* contributes to neurotransmitter release, and shapes behavioral responses to stimuli in  
354 *D. melanogaster*. In particular, *FMRFaR* plays a role in muscle contraction and larval response  
355 to light (Ravi et al. 2018; Klose et al. 2010). *AmGR10* is a gustatory receptor in *Apis mellifera*  
356 and disrupting its activity affects the division of labor within honeybee hives (Paerhati et al.  
357 2015). Specifically, this gene is expressed in nurses, and knockdown of gene expression causes  
358 nurses to transition to foraging (Paerhati et al. 2015). Recent functional characterization of  
359 *AmGR10* in *A. mellifera* reveals that this gene is a broadly-tuned amino acid receptor (Lim et al.  
360 2019). This gene overlaps with the trans-species, haplotype-specific SNPs detected in our  
361 comparisons (Fig. 3C), while two other genes are upstream and downstream of this area (Table  
362 3). Further work is needed to examine the specific functions of these loci in *Formica* ants. One  
363 major challenge in understanding the genetic underpinnings of colony-level traits, such as queen  
364 number, is that these traits are difficult to assess in a standard gene knockdown assay.

365

#### 366 *Additional patterns in the phylogenetic dataset*

367 Several additional patterns are visible in the comparison of *knockout* SNPs (Fig. 1). First,  
368 in comparing the outgroup genotypes, we note that *Iberoformica* is homozygous for the reference  
369 allele at 19 out of 20 sites, while *Polyergus* exhibits a mix of reference and alternate alleles.  
370 While preliminary, this finding suggests that the Sm form of *knockout* may have been present in  
371 the common ancestor of *Formica* and *Iberoformica*. This pattern was detected at the previously-  
372 identified trans-species, haplotype-specific SNPs within *knockout*, but not in other parts of the  
373 chromosome (Table S3). Second, samples from three of the 14 clades examined here contained  
374 exclusively Sm/Sm homozygous individuals. We only sequenced three samples from the  
375 *dakotensis* clade and four samples from *fuscus* clade #6, but all 10 *difficilis* clade individuals were



376 homozygous for the *F. selysi* Sm/Sm reference alleles at *knockout*. Members of this clade are  
377 socially parasitic and include temporary social parasites and at least one permanent inquiline of  
378 other *Formica* species (Talbot 1976; Wilson 1976; Buschinger 1986; Borowiec et al. 2021).  
379 Previous research has suggested that polygyny can be a preadaptation for social parasitism  
380 (Alloway 1980; Elmes 1980; Bourke & Franks 1991). It's noteworthy that members of the  
381 *difficilis* clade are facultatively polygynous and evolved from a facultatively polygynous  
382 common ancestor (Borowiec et al. 2021) but that extant *difficilis* clade species may lack the  
383 supergene haplotype that is associated with polygyny in at least five congeneric species  
384 (Brelsford et al. 2020).

385         In the *neogagates-pallidefulva* clade, we detected a third haplotype at the *knockout* locus  
386 (Fig. 1). Given that these samples otherwise appear to be heterozygous at other trans-species,  
387 haplotype-specific SNPs detected within the *neogagates-pallidefulva* clade (Fig. 2A), we  
388 hypothesize that this haplotype reflects a clade-specific gene conversion or recombination event,  
389 and that most conserved, functionally relevant SNPs are within the 11,910,356-11,910,880 bp  
390 window. Finally, we note the dearth of samples that are apparently homozygous for the  
391 polygyne-associated haplotype at *knockout*. While Sp/Sp homozygotes are common in *F. selysi*  
392 (Purcell et al. 2014; Avril et al. 2019) and close relatives *F. cinerea* and *F. lemni* (Brelsford et  
393 al. 2020), our current sampling effort, which focused on maximizing species diversity, resulted  
394 in the addition of only one putative Sp/Sp homozygote in *fusca* clade #4. Samples were collected  
395 by several researchers without attention to colony social structure, so this pattern may simply  
396 reflect biased sampling. Alternatively, we speculate that the rarity of Sp/Sp in this sample raises  
397 the possibility that the Sp haplotype has experienced degeneration in some *Formica* lineages,  
398 similar to that observed in the *Solenopsis* supergene (Pracana et al. 2017; Stolle et al. 2019).

399

400 *Limitations and future directions*

401 Our study has several limitations that could influence the location and number of trans-  
402 species, haplotype-specific SNPs identified, but that are unlikely to affect the overall pattern that  
403 we described. We avoided assessing haplotype-specific variation on chromosome 3 for clades  
404 represented by few genomes because the small samples size could inflate the numbers of SNPs  
405 that show a false positive difference between alternative haplotypes. A subset of the trans-  
406 species, haplotype-specific SNPs identified within single clades (Figs. 2D-F), for example, are  
407 likely to be false positives. As we include more species spanning a deeper evolutionary history,  
408 our metric of assessing trans-species, haplotype-specific SNPs becomes highly conservative. In  
409 this analysis, we only include loci that show a perfect association with the *knockout* haplotype.  
410 Genotyping errors will inflate the number of false negatives, and this tendency will increase as  
411 the sample size grows. Finally, we aligned reads from all genomes to the *Formica selysi*  
412 reference genome (Brelsford et al. 2020), which is based upon an individual with the Sm/Sm  
413 genotype. While most sequences aligned to the reference genome, rapid divergence at some  
414 genomic regions could impede alignment for more distantly related species. All of our  
415 alignments assume the Sm haplotype orientation, which is collinear between *F. selysi* and *F.*  
416 *exsecta* (Brelsford et al. 2020). However, we know that there have been multiple rearrangements  
417 of chromosome 3 between Sm and Sp haplotypes in *F. selysi*. As a result, we cannot draw  
418 conclusions about the relative positions of the trans-species, haplotype-specific SNPs in all  
419 species or on both haplotypes. A useful complement to this study and future direction will be to  
420 examine structural variation on chromosome 3 across the genus *Formica*.

421 *Conclusion*

422 Overall, we demonstrate that genetic polymorphisms associated with variation in colony  
423 queen number in at least some *Formica* species (Purcell et al. 2014; Brelsford et al. 2020) likely  
424 predate the common ancestor of all extant *Formica*. Using a comparative approach, we have  
425 pinpointed additional genes within the supergene region that harbor trans-species, haplotype-  
426 specific SNPs in an early diverging, Nearctic *Formica* clade.

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436  
437 **Data Accessibility and Benefit-Sharing Statement:** Sequences generated for this study will be  
438 placed on the NCBI SRA. Genotypes used in the analyses presented in this manuscript are  
439 provided in the supplementary materials files, in tables S2 and S3.

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440  
441 **Author contributions:** JP and AB conceived of the study. JP, MLB, CR, and AB collected and  
442 solicited the samples, MLB and CR identified the samples, and JP, MLB, and AB performed lab  
443 work to prepare samples for sequencing. AB and GLR analyzed the data. JP wrote the  
444 manuscript with advice from all authors.

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## 621 Figures and Tables

622 **Fig. 1:** The 22 SNPs with fixed differences between Sm and Sp haplotypes at the gene *knockout*  
623 that were conserved in 15 European species (Brelsford et al. 2020) are also conserved in North  
624 American *Formica* species. *Formica* species in our dataset cluster into 14 clades based on a  
625 neighbor joining tree. This tree aligns well with the best current *Formica* phylogeny, produced  
626 by Borowiec et al. (2021). Key divergence dates inferred by Borowiec et al. are indicated in  
627 grey circles on several nodes: node 1 is ~23–40 Ma, node 2 is ~20–30 Ma, and node 3 is ~17–27  
628 Ma. Genotypes matching the reference genome (*F. selysi* Sm/Sm) are shown in green.  
629 Heterozygous loci are shown in light orange and loci homozygous for the alternate allele  
630 ('Sp/Sp') are shown in dark orange. White boxes represent missing data. Each clade is  
631 represented by a unique color bar (right), which we use consistently in other tables and figures to  
632 denote clade (Table S1, Fig. 2). Individual genomes that were previously analyzed by Brelsford  
633 et al. (2020) are shown in blue (monogyne origin), red (polygyne origin), and grey (social  
634 structure unknown) highlights over the species name.  
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638 **Fig. 2:** Comparing the five *knockout* heterozygotes and six *knockout* homozygotes from the  
639 *neogagates-pallidefulva* clade (A), we detect 429 clade-level trans-species, haplotype-specific  
640 SNPs. The majority of these are distributed from 2–12.5 Mbp along chromosome 3 with several  
641 notable peaks of divergence between alternative haplotypes at 2.3, 11.6, 11.9, and 12.4 Mbp.  
642 Species in this comparison share a common ancestor dating to about 24 Ma (Borowiec et al.  
643 2021). We compare individuals with alternative *knockout* variants across the socially parasitic  
644 *Formica* lineages (B), which share a single common ancestor dating to about 18 Ma. We find  
645 peaks of divergence again at 2.3 and 11.6 Mbp, as well as many other clade-specific peaks.  
646 Comparing species from three *fusca* clades (C), we find only 68 trans-species, haplotype-specific  
647 SNPs. The majority of these SNPs are found at 2.3, 10.3, 11.6, and 11.9 Mbp (the latter includes  
648 *knockout*). These three clades share a common ancestor dating to about 17 Ma. We then  
649 investigate the distribution of trans-species, haplotype-specific SNPs in each of the three clades  
650 separately (D–F) to determine whether the patterns are consistent with the predictions of the  
651 'eroded strata' model (Table 1). For *fusca* clade #3 (D, common ancestor dates to about 12 Ma),  
652 which includes *F. selysi*, we find clusters of trans-species, haplotype-specific SNPs spanning the  
653 2–12.5 Mbp region containing the 'social' supergene (Purcell et al. 2014). We find a similar  
654 pattern in *fusca* clade #4 (E, common ancestor dates to about 13 Ma), except that we detect low  
655 levels of trans-species, haplotype-specific SNPs across the full extent of the chromosome. We  
656 find fewer SNPs in *fusca* clade #5 (F, common ancestor dates to about 14 Ma), with clear peaks  
657 of divergence at 2.3, 10.3, 11.6, and 11.9 Mbp. The colors of the data points reflect clade-  
658 specific colors (Fig. 1, Table S1).  
659

660 **Fig. 3:** Trans-species, haplotype-specific SNPs that are present across four well-sampled clades,  
661 including *neogagates-pallidefulva*, *exsecta*, *rufa*, and *fusca* clade #3 are found in several clusters  
662 along chromosome 3 (A). In the latter three clades, we are comparing Sp males and Sp/Sp  
663 workers with Sm males and Sm/Sm workers. Some peaks of divergence, most notably the



664 region at 2.3 Mbp, show an unusual pattern of apparent heterozygosity in the Sp haploids,  
665 suggesting possible gene duplications in these regions (circles). We also detected some peaks of  
666 divergence that appear to include gene duplications in only one clade, *fusca* clade #3 (squares).  
667 There are relatively few trans-species, haplotype-specific SNPs that never appear heterozygous  
668 in the Sp males (triangles). For the regions with the greatest number of trans-species, haplotype-  
669 specific SNPs, we show zoomed in views of the SNPs near the genes *single-minded* (B) and  
670 *AmGR10* (C). Exons in these genes are shown as gray bars (B and C). The arrow below each  
671 figure indicates the orientation of the gene. We indicate the positions of the seven genes  
672 identified in Table 3 (A).

673 **Table 1:** Predictions of and empirical evidence consistent with the eroded strata model at three distinct time points. Visual predictions  
 674 of this verbal model and additional background are presented by Brelsford et al. (2020, Figure 4).

Comparison	Expected pattern	Empirical evidence consistent with the prediction	Additional expectations
Within polymorphic species	Suppressed recombination results in differentiation between alternative haplotypes across the length of the supergene.	$F_{ST}$ between individuals from single- and multiple-queen colonies is elevated from 2-~12.5 Mbp along chromosome 3 in five species (Brelsford et al. 2020, Figure 1). Throughout this 10.5 Mbp supergene region, linkage disequilibrium is high in <i>F. selysi</i> (Figure 2B in Purcell et al. 2014).	Recent recombination or gene conversion events may be detected as a polymorphism within one haplotype. An example was observed in the <i>F. selysi</i> Sp haplotype (Purcell et al. 2014, Figure S1J).
Between closely related polymorphic species	Comparing alternative trans-species haplotypes will reveal many SNPs that are shared across species. There may be some areas within the region of suppressed recombination that lack such SNPs.	The related species <i>F. selysi</i> , <i>F. cinerea</i> , and <i>F. lemni</i> share trans-species, haplotype-specific SNPs along long stretches of the supergene (Brelsford et al. 2020, Figure 3). Within relatively young clades ( <i>fusca</i> clades #3, 4, and 5), we similarly see trans-species, haplotype-specific SNPs spanning most of chromosome 3 (this study, Figures 2D-F).	Clades with a longer evolutionary history or more representatives in the analysis will have smaller regions harboring trans-species, haplotype-specific SNPs within the region of suppressed recombination.
Between more distantly related groups of polymorphic species.	As the trans-species analysis is expanded to include groups sharing an increasingly ancient common ancestor, the comparison will encompass a larger number of rare, branch-specific recombination events if we assume that rare recombination occurs at a slow and steady rate after the inversion forms. These regions will not harbor trans-species, haplotype-specific SNPs. Such SNPs will only be found in parts of the chromosome that have remained differentiated between the alternative haplotypes in all branches of the tree.	When comparing across the genomes of 15 European <i>Formica</i> species, Brelsford et al. (2020) found 22 trans-species, haplotype-specific SNPs in the gene <i>knockout</i> (Brelsford et al. 2020, Figure 1). Here, we greatly expand trans-species analyses of the SNPs in <i>knockout</i> (this study, Figure 1), as well as SNPs across chromosome 3 in the <i>neogagates-pallidefulva</i> clade (Figure 2A), the socially parasitic clade (Figure 2B), and across <i>fusca</i> clades #3-5 (Figure 2C).	At this scale, we expect the trans-species, haplotype-specific SNPs to either be localized in parts of the chromosome that are under selection or in areas that cannot recombine due to structural constraints (e.g. inversion breakpoints). We note that there may also be differing structural rearrangements between alternative haplotypes in different clades.

675 **Table 2:** Genome sequencing libraries were prepared in three groups, as shown here. Details  
 676 about each sample are shown in table S1.

Number of genomes	Source	Library preparation method	Depth (range)
21	Brelsford et al. 2020	TruSeq	7-18.2x
15	collected by Purcell and Brelsford	TruSeq	5.3-15x
1	collected by Purcell and Brelsford	Nextera	8.7x
80	collected by Borowiec, Cover and Rabeling	Kapa	1.5-30.7x

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**Table 3:** Regions of the supergene that harbor large numbers of trans-species, haplotype-specific SNPs overlap with several genes that could play a functional role in the evolutionary origin of the supergene or in the contemporary maintenance of polymorphism. We briefly summarize the functions of these genes in model systems when possible.

gene	ortholog name	trans-species SNP position(s)	putative function	notes	citation
<i>Knockout (Drosophila melanogaster)</i>	STOX1 ( <i>Homo sapiens</i> )	Scaffold03: 11,910,116-11,911,137	motor neuron development	conserved trans-species fixed SNPs detected in 11/14 <i>Formica</i> clades	Brelsford et al. 2020; Hartmann et al. 1997
<i>Single-minded (D. melanogaster)</i>	SIM1 ( <i>H. sapiens</i> )	Scaffold03: 2,261,463-2,275,100	neurogenesis, transcription	Sp version appears to contain a duplication (Fig. 3)	this article; Umetsu et al. 2006
<i>AmGR10 (Apis mellifera)</i>	NA	Scaffold03: 11,647,776-11,683,774	gustatory receptor, implicated in division of labor	<i>F. exsecta</i> annotation may have incorrectly split this gene in two	this article; Paerhati et al. 2015; Lim et al. 2019
<i>tplus3b (D. melanogaster)</i>	<i>mapk15 (H. sapiens)</i>	Scaffold03: 11,647,776-11,683,774	ATP binding, DNA and telomere repair, regulation of autophagy	function examined in humans, only the last intron overlaps with conserved trans-species fixed SNPs	this article; Colecchia et al. 2012, Klevernic et al. 2009
<i>zinc finger protein 148-like</i>	NA	Scaffold03: 11,647,776-11,683,774	transcription regulation	just upstream of putative AmGR10	this article
<i>FMRFaR (D. melanogaster)</i>	NA	Scaffold03: 12,353,285-12,369,100	neuropeptide receptor, locomotion behavior, larval response to light	part of this region may have been duplicated on Sp haplotype in some clades (Fig. 3)	this article; Ravi et al. 2018; Klose et al. 2010
<i>Uncharacterized protein (LOC115241360)</i>	NA	Scaffold03: 10,262,710-10,263,020	possible transcription factor		this article

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