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Linked supergenes underlie split sex ratio and social organization in an ant

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20 **Abstract**

21 Sexually reproducing organisms usually invest equally in male and female offspring.
22 Deviations from this pattern have led researchers to new discoveries in the study of
23 parent-offspring conflict, genomic conflict, and cooperation. Some social insect species
24 exhibit the unusual population-level pattern of split sex ratio, wherein some colonies
25 specialize in the production of future queens and others specialize in the production of
26 males. Theoretical work focused on the relatedness asymmetries emerging from
27 haplodiploid inheritance, whereby queens are equally related to daughters and sons,
28 but their daughter workers are more closely related to sisters than to brothers, led to a
29 series of testable predictions and spawned many empirical studies of this phenomenon.
30 However, not all empirical systems follow predicted patterns, so questions remain about
31 how split sex ratio emerges. Here, we sequence the genomes of 138 *Formica glacialis*
32 workers from 34 male-producing and 34 gyne-producing colonies to determine whether
33 split sex ratio is under genetic control. We identify a supergene spanning 5.5 Mbp that
34 is closely associated with sex allocation in this system. Strikingly, this supergene is
35 adjacent to another supergene spanning 5 Mbp that is associated with variation in
36 colony queen number. We identify a similar pattern in a second related species,
37 *Formica podzolica*. The discovery that split sex ratio is determined, at least in part, by a
38 supergene in two species opens a new line of research on the evolutionary drivers of
39 split sex ratio.

40 **Significance Statement**

41 Some social insects exhibit split sex ratio, wherein some colonies produce future
42 queens and others produce males. This phenomenon spawned many influential
43 theoretical studies and empirical tests, both of which have advanced our understanding
44 of parent-offspring conflicts and cooperation. However, some empirical systems did not
45 follow theoretical predictions, indicating that researchers lack a comprehensive
46 understanding of the drivers of split sex ratio. Here, we show that split sex ratio is
47 associated with a large genomic region in two ant species. The discovery of a genetic
48 basis for sex allocation in ants provides a novel explanation for this phenomenon,
49 particularly in systems where empirical observations deviate from theoretical
50 predictions.

51

52 Introduction

53 The relative investment in male versus female offspring is a vital fitness
54 component of sexually reproducing organisms. Research on sex allocation theory has
55 yielded breakthroughs in our understanding of topics as diverse as parent-offspring
56 conflict, evolution of cooperation, and genomic conflict (1). Parent-offspring conflict is
57 predicted to occur in subdivided populations with strong local mate competition, as seen
58 in polyembryonic parasitoids (2), and in systems with relatedness asymmetry between
59 sisters and brothers, as found in haplodiploid species such as the primitively eusocial
60 wasp *Polistes chinensis antennalis* (3). Parental control of sex ratio is also thought to
61 contribute to the maintenance of cooperative breeding; for example, Seychelles
62 warblers living in high quality territories where helpers provide strong benefits produce
63 an excess of females, the helping sex (4). However, similar patterns of biased sex
64 allocation increasing the frequency of the helping sex are not found among all
65 cooperatively breeding birds (5). The discovery of a chromosome that skews sex ratio
66 from female-biased to 100% male in the jewel wasp *Nasonia vitripennis* was the first
67 clear empirical illustration of an intragenomic conflict (6). This paternal sex ratio
68 chromosome is transmitted through sperm to fertilized eggs, where it causes the loss of
69 other paternally inherited chromosomes to produce exclusively male offspring (7, 8).
70 Subsequent discoveries of sex ratio distorter systems take different forms, including
71 female biased sex ratios mediated by endosymbionts (9, 10). These studies opened the
72 door for additional research on intragenomic conflict in multiple contexts, including
73 between sexes (11, 12) and between social insect castes (13).

74 Where there is intragenomic conflict, one resolution is evolution of suppressed
75 recombination to reduce the frequency of deleterious multilocus genotypes. This is
76 illustrated in the standard model of sex chromosome evolution (14, 15), in which
77 selection favors the loss of recombination between a sexually antagonistic locus and a
78 sex-determining locus on the same chromosome, eventually leading to a Y or W
79 chromosome that is exclusively present in one sex. Under the “reduction principle” (16),
80 this is also expected to occur around sex-ratio distorters. In line with this prediction, sex-
81 ratio distorter loci often occur in regions of low recombination (17–20), but we lack
82 evidence for the direction of causality. The reduction principle is also expected to

83 contribute to the formation of autosomal supergenes controlling other complex traits that
84 involve epistatic interactions between two or more loci. Such supergenes have been
85 found to control phenotypes including polymorphic wing coloration in butterflies, mating
86 strategies in birds and fungi, self-incompatibility in plants, and colony social organization
87 in ants (21–28). Autosomal supergenes, like sex chromosomes, are likely to represent
88 the resolution of past intragenomic conflict between two or more loci.

89 Supergenes underlie at least two independently evolved cases of social
90 polymorphism in ants. In the fire ant *Solenopsis invicta*, colony queen number is
91 controlled by a supergene spanning most of a single chromosome (27). *Formica selysi*
92 has a similar chromosome-spanning supergene underlying colony queen number, but
93 there is no detectable overlap in gene content between the two (28). More recently, both
94 ant social supergenes were shown to underlie colony queen number in other congeneric
95 species (29, 30). In both systems, the haplotype associated with multi-queen (=
96 polygyne) social structure is a selfish transmission distorter (31–33). These discoveries
97 raise new questions about links between social structure and sex ratio that have been
98 proposed in classic literature about sex allocation in Hymenoptera.

99 Trivers and Hare (34) proposed that queen-worker conflict, which is shaped by
100 relatedness asymmetry within each nest, drives biased sex ratios. Since workers are
101 more related to their full sisters (average relatedness = 0.75) than to their brothers
102 (average relatedness = 0.25), workers in single-queen, monandrous colonies should
103 favor the production of queens over males. Trivers and Hare (34) predicted that worker
104 interests would prevail in these cases, resulting in female-biased offspring production.
105 Queens are equally related to male and female offspring, so they should generally favor
106 a 1:1 sex ratio. In colonies with multiple queens or multiple mates, the low relatedness
107 reduces this conflict between queens and workers, resulting in weaker selection for
108 biased sex allocation (34). Although these predictions revolutionized the way that
109 researchers think about fitness and relatedness in social insect colonies, they are not
110 ubiquitously upheld in empirical studies (1).

111 Strikingly, some social insect species exhibit a nearly complete segregation of
112 male and queen production at the colony level, in a phenomenon known as 'split sex
113 ratio'. This extreme case has been observed in at least 20 different genera of ants,

114 wasps, and bees (35–37). Boomsma and Grafen (36) argued that this pattern is
115 consistent with worker control of sex ratio in populations with variation in relatedness
116 asymmetry: workers that are more related than the population average to their
117 nestmates should favor specializing in the production of new queens (hereafter
118 "gynes"), while those that are less related than average should specialize in male
119 production (36, 38). The variation in relatedness asymmetry would emerge from the
120 number of mates per queen and from the number of queens per colony.

121 The models of Boomsma and Grafen inspired a burst of empirical research on
122 split sex ratio in ants and other social insects (37, 39–45, 45–53). Ants in the genus
123 *Formica* emerged as a prominent model system, as a result of their widespread and
124 well documented variation in sex ratio and social structure (35). Many species exhibit
125 split sex ratio or highly biased sex ratio (34, 41–45), but not all of these examples follow
126 predicted patterns based on relatedness asymmetry. Finnish populations of *Formica*
127 *truncorum* and *F. exsecta* follow theoretical predictions: in colonies with a single queen
128 (= monogyne), monandrous queens tend to produce gynes, while polyandrous queens
129 tend to produce males (41, 44, but see 46). A similar pattern was found in monogyne
130 and polygyne colonies in *F. truncorum* (with polygyne colonies producing males (45)). A
131 socially polymorphic population of *F. selysi* and a polygynous population of *F. exsecta*
132 that exhibits variation in relatedness asymmetry deviated from these predicted patterns
133 (46, 48). Additional studies have identified potential roles of habitat and diet in shaping
134 sex allocation in *F. podzolica* (44), in *F. exsecta* (49), in *F. aquilonia* (50), as well as
135 colony needs for queen replacement (51, 55). Finally, although *Wolbachia* is present in
136 some *Formica* species exhibiting split sex ratio, it does not appear to influence sex ratio
137 in any system studied so far (52, 53).

138 Taken together, it appears that there are yet missing pieces to the puzzle of how
139 and why ants achieve a split sex ratio. A meta-analysis attributed only ~25% of the
140 observed variance in sex allocation to relatedness asymmetry and variation in queen
141 number (37). Theoretical examinations following from this finding support a possible role
142 for virgin queens (which would produce only male offspring) or queen replacement (56),
143 but another possibility is that *sex allocation by queens is itself under genetic control*.

144 Here, we examine the evidence for this mechanism, which could be responsible
145 for much of the unexplained variance in patterns of split sex ratio. We 1) conduct a
146 genome-wide association study for variants associated with sex ratio in *Formica*
147 *glacialis*, 2) infer transmission patterns of sex-ratio-associated variants from colony-level
148 genotype frequencies, 3) evaluate whether sex ratio and social organization map to the
149 same region of the genome, and 4) test for a shared genetic basis of sex ratio in the
150 related species *F. podzolica*.

151 Results

152 Through a genome-wide association study (GWAS) of 138 *F. glacialis* whole-
153 genome sequences, we identify numerous variants associated with colony sex
154 allocation in a region of chromosome 3 spanning 5.5 Mbp (Figure 1a). A principal
155 component analysis (PCA) of variants on chromosome 3 reveals three distinct genotype
156 clusters, one of which is observed in just six individuals (Fig 1b). Of the workers with
157 low PC2 scores (yellow and green clusters, Fig. 1b) 60.2 % were collected from male-
158 producing colonies, while 93.3% of workers with high PC2 scores (purple cluster, Fig.
159 1b) were from gyne-producing colonies. We investigate the parts of chromosome 3
160 that distinguish the genotype clusters through an assessment of F_{ST} between the
161 clusters (Fig. 2). This comparison reveals two adjacent regions of differentiation.
162 Between the two clusters with low PC1 scores, we observe differentiation spanning the
163 region from 2-7.5 Mbp (Fig. 2a), similar to the region revealed in the initial GWAS.
164 Between the two clusters with low PC2 values (both of which harbored an excess of
165 workers from male producing colonies), we identify a differentiated region from about
166 7.5-12.5 Mbp, as well as a small peak at 2 Mbp (Fig. 2b).

167 Previous studies found that colony queen number in *F. selysi* (28, 57) and other
168 European *Formica* species (29) is controlled by a social supergene on chromosome 3.
169 To determine whether a supergene on chromosome 3 similarly underlies colony queen
170 number in *F. glacialis*, we investigated variation in 19 additional colonies from other
171 populations using ddRADseq. Opposing homozygosity among nestmates (i.e. the
172 presence of two alternative homozygous genotypes in nestmates, which is not possible
173 in haplodiploid full siblings) reveals substantial variation in colony social structure in this
174 species (Fig. 3a), and this variation maps to the supergene region (Fig. 3b). In

175 particular, SNPs that are significantly associated with variation in social structure in the
176 ddRADseq data are localized in the 7.5-12.5 Mbp region (Fig. 3c), corresponding to the
177 region identified in Fig. 2b.

178 A PCA of markers on chromosome 3 that are shared in the whole genome and
179 ddRADseq datasets reveals that the colonies that are assessed to be polygyne based
180 on a high frequency of opposing homozygosity (red, Fig. 3d) consistently harbor one
181 genotype. This genotype is shared with the six individuals from the whole genome
182 sequencing library that formed the yellow cluster in Fig. 1b. These individuals are
183 heterozygous for two alternative supergene haplotypes, one of which appears to occur
184 exclusively in polygyne colonies. We define this haplotype as the Sp haplotype of *F.*
185 *glacialis*. We note that the Sp found in other *Formica* species spanned about 10.5 Mbp
186 of chromosome 3, from 2 Mbp to about 12.5 Mbp (29), while the one identified here in *F.*
187 *glacialis* is shorter. The remaining two genotype clusters identified in the whole genome
188 dataset (green and purple clusters, Fig. 1b) both group with workers from colonies
189 assessed to be monogyne in the ddRADseq dataset based on very low levels of
190 opposing homozygosity (Fig. 3a). Based on the regions of differentiation among
191 genotype clusters (Fig. 2), we hypothesized that individuals from the purple cluster carry
192 two alternative supergene haplotypes in the 2-7.5 Mbp region of chromosome 3
193 (subsequently confirmed with PCR-RFLP genotyping; Fig. 4). One of these haplotypes
194 is found almost exclusively in gyne-producing colonies. The other haplotype is usually
195 homozygous in male-producing colonies. Since one genotype is associated with the
196 production of daughters in monogyne colonies, we name these alleles after
197 mythological twins Danaus and Aegyptus, who had 50 daughters and 50 sons,
198 respectively. Individuals from the gyne-producing cluster (purple, Fig. 1b) have the
199 genotype Sma/Smd, while those from the predominantly male-producing cluster have
200 the genotype Sma/Sma (green, Fig. 1b).

201 We developed two PCR-RFLP assays to distinguish these three genotypes in a
202 larger number of individuals from each of the colonies in the focal population in Yukon
203 Territory. Workers from gyne-producing colonies are a mix of Sma/Smd heterozygotes
204 and Sma/Sma homozygotes, while workers from male-producing colonies are most
205 often Sma/Sma homozygotes or Sp/Sma heterozygotes (Fig. 4a). This suggests that

206 gyne-producing monogyne colonies are usually headed by Sma/Smd queens, while
207 male producing monogyne colonies are usually headed by Sma/Sma queens (Fig. 4b).
208 Looking at each colony, we show that 31 out of 34 gyne-producing colonies harbor at
209 least one Sma/Smd worker out of eight genotyped, while 27 out of 34 male-producing
210 colonies harbor only Sma/Sma workers and Sma males (Fig. 4c). Among the remaining
211 male-producing colonies, three harbor only Sp/Sma workers (and are likely polygyne).
212 Of these, two had Sp males and one had a single Sma male. Four male-producing
213 colonies host a mix of Sma/Sma and Sma/Smd workers, as well as both Sma and Smd
214 males. We infer the genotypes of individuals from colonies with known social structure
215 in the ddRADseq dataset using a set of diagnostic SNPs. Across these additional
216 populations, we show that two monogyne colonies harbor exclusively Sma/Sma
217 workers, while nine harbor a mix of Sma/Smd and Sma/Sma workers (Fig. 4d). The
218 four polygyne colonies all contain Sp/Sma workers; one colony contains a single
219 Sp/Smd worker as well.

220 We obtained a smaller sample of colonies of *F. podzolica*, the sister species of *F.*
221 *glacialis*, that exhibited a split sex ratio at the focal site in the Yukon Territory. While the
222 GWAS analysis is inconclusive (Fig. S1), we observe similar qualitative patterns in the
223 genomic differentiation between genotype clusters identified in a PCA (Fig. 5).
224 Individuals from these two PCA clusters (Fig. 5a) exhibit elevated genetic differentiation
225 from 2-7.5 Mbp along chromosome 3 (Fig. 5b). Gyne-producing colonies harbor a mix of
226 putative Sma/Smd heterozygotes and Sma/Sma homozygotes. The majority of male-
227 producing colonies contain exclusively Sma/Sma workers (Fig. 5c). A large number of
228 SNPs distinguishing Sma and Smd haplotypes are conserved between *F. podzolica* and
229 *F. glacialis* (Fig. 5d).

230 Discussion

231 We demonstrate that a chromosome underlying queen number across the
232 *Formica* genus is also associated with the split sex ratio patterns observed in a sister
233 species pair. Sex ratio variation based on queen genotype could account for the many
234 empirical exceptions (37) to the patterns predicted by Boomsma and Grafen (36, 38). In
235 *Formica glacialis*, we show that the Smd supergene haplotype behaves like a 'W' sex
236 chromosome in that it's present almost exclusively in females and in a heterozygous

237 state. A key difference is that it influences the sex ratio of offspring rather than the sex
238 of the individual bearer. Single-queen gyne-producing colonies generally harbor a mix
239 of Sma/Smd and Sma/Sma workers, suggesting that the queens are Sma/Smd
240 heterozygotes crossed with Sma males. Through Mendelian inheritance, half of their
241 daughters (the heterozygotes) will in turn be gyne-producing queens, while the other
242 half will be male producers. Males are produced either by homozygous Sma/Sma
243 single queens or by polygyne (Sp/Sma) queens. We noted a few exceptions to this
244 pattern in both gyne- and male-producing colonies, suggesting that genetic control is
245 imperfect. Our focal population was predominantly monogyne, so we do not yet know if
246 some polygyne colonies specialize in producing gyns.

247 A striking finding of this study is that the extent of the social supergene
248 discovered in other *Formica* species appears to be split into two adjacent, linked
249 supergene regions in *F. glacialis*. One half of the supergene, from 2-7.5 Mbp on
250 chromosome 3, is associated with split sex ratio. The other half, from 7.5-12.5 Mbp,
251 which includes the gene *knockout* identified as a candidate conserved gene influencing
252 social structure in other *Formica* species (29), is associated with social structure (Fig.
253 2).

254 These patterns raise questions about the evolution of the functions of these two
255 linked supergenes in *F. glacialis* and *F. podzolica*. Theory predicts split sex ratio to
256 evolve in social hymenopteran populations with variation in relatedness asymmetry. We
257 propose two alternative scenarios that could explain the evolution of these linked
258 regions. In one scenario, we speculate that split sex ratio may have evolved in socially
259 polymorphic *Formica* populations, wherein monogyne and monandrous queens would
260 specialize in gyne production, while polygyne or polyandrous queens would produce
261 predominantly males. Such patterns were documented in other *Formica* species,
262 including *F. truncorum* (42, 58) and Finnish populations of *F. exsecta* (54), although we
263 note that this pattern is not present in all previously studied *Formica* species (46, 51).
264 Specialization in offspring sex ratio based on social structure would select for reduced
265 recombination between loci influencing sex ratio and social structure. In populations
266 with little relatedness asymmetry, as observed in our predominantly monogyne *F.*
267 *glacialis* population in the Yukon, rare recombinant supergene haplotypes that decouple

268 social determination from sex ratio determination could spread in the population. In this
269 case in particular, we suggest that gene flux from the Sp haplotype onto the Sm may
270 have led to the formation of the Sma haplotype associated with monogyne social
271 structure and the production of males. Such sex ratio supergene systems may persist in
272 species with a mix of socially polymorphic and socially monomorphic populations, which
273 could explain deviations from the theoretical predictions of Boomsma and Grafen (36),
274 wherein predominantly polygyne populations produce highly male biased sex ratios, as
275 in Swiss *F. exsecta* (48), or one social form exhibits strongly split sex ratios and the
276 other is intermediate, as in *F. selysi* (46).

277 In an alternative scenario, a gene or supergene influencing sex ratio could
278 predate the appearance of persistent social polymorphism; when alternative social
279 structures emerged, selection for male-biased production in colonies with lower average
280 relatedness and for gyne-biased production in colonies with higher average relatedness
281 could have led to the appearance of linked genetic variants favoring one or more
282 queens. The dual roles of linked supergenes in shaping social organization and sex
283 ratio in *Formica* species could help to explain why this supergene has persisted for
284 millions of years (29). Future studies could examine these speculative scenarios by
285 seeking evidence of sex ratio supergenes in other, distantly related *Formica* species. In
286 particular, we predict that a supergene like this one could be particularly evident in
287 stable populations with little relatedness asymmetry.

288 Our study in *F. glacialis* is not the first to identify sex-specific genetic differences
289 between ant gynes and males (20, 59). However, the mechanisms that produce these
290 sex-specific genetic differences appear to differ across systems, and none are fully
291 understood. Kulmuni and Pamilo (59) showed that hybridization between *Formica*
292 *aquilonia* and *Formica polyctena* results in admixed females, but that surviving males
293 tend to have a genotype comprised of alleles from only one parental species (59, 60).
294 They proposed that recessive incompatibilities between the genomes of the two species
295 are exposed to selection in haploid males. Subsequent research on this system has
296 revealed instability in the direction of selection on introgressed alleles in males across a
297 ten-year time interval, with introgression recently favored on average in loci where
298 introgression was previously selected against (61). In the tawny crazy ant *Nylanderia*

299 *fulva*, males invariably carry the same allele at two out of 12 microsatellite loci, while
300 females are almost always heterozygous at these loci (20). Diploid eggs that were
301 homozygous for the male-associated alleles at these loci failed to develop. In *F.*
302 *glacialis*, we similarly observe a haplotype, Smd, that is found almost exclusively in
303 females. However, we speculate that the mechanism underlying the sex-specific genetic
304 differences in *F. glacialis* may not rely on lethal effects of alleles in one sex or the other,
305 as it appears to in the aforementioned systems. We suggest that the Sma/Smd queens
306 lay exclusively fertilized eggs, which would preclude the production of males. Further
307 research is needed in all systems to compare the specific mechanisms maintaining
308 these genetic differences between the sexes.

309 Some previous empirical discoveries still need further examination in the light of
310 the newly discovered split sex ratio supergene. Several experimental studies provided
311 evidence that environmental quality and diet can influence colony sex ratio, including in
312 a population of *F. podzolica* from central Alberta (44). We posit that there may be as yet
313 undetected gene x environment interactions, which could explain the rare deviations
314 from expected genotype distributions in workers from our gyne- and male-producing
315 colonies. Repeating food supplementation experiments in colonies of known genetic
316 structure will help to resolve this question. Although the linkage between social and sex
317 ratio supergenes hints at a role of parent-offspring conflict in shaping split sex ratio in
318 *Formica* ancestors (34, 38), many questions remain about how worker control could
319 function in a system with genetic determination of sex ratio. Understanding how the sex
320 ratio supergene functions will help to illuminate how the contemporary conflict plays out.
321 For example, does the Smd haplotype cause cessation of haploid egg production?
322 What factors prevent female offspring of Sma/Sma queens from developing into gynes
323 instead of workers?

324 Despite limited sampling, we also document an intriguing deviation in the mode
325 of action of the *F. glacialis* social supergene compared to that of *F. selysi*. Across the
326 individuals in the RADseq dataset collected from polygyne colonies, we did not detect
327 any Sp/Sp homozygous individuals (Fig. 4). Polygyne colonies harbored almost
328 exclusively Sp/Sma workers (N = 30), with only a single Sp/Smd worker. In contrast,

329 polygyne *F. selysi* colonies contain exclusively Sp/Sm and Sp/Sp workers and Sp males
330 (28, 33, 57). We did not detect systematic variation at the supergene in monogyne *F.*
331 *selysi* workers (28, 57), but we note that analyses were carried out with relatively sparse
332 RADseq markers, so it is possible that a small sex ratio supergene could be present in
333 *F. selysi*.

334 Here, we describe a supergene that drives offspring sex ratio in ants. This sex
335 ratio supergene is closely linked with a previously described supergene that underlies
336 colony queen number in *Formica* ants. The discovery that split sex ratio has a genetic
337 basis helps to resolve the conflicting empirical results about whether and how split sex
338 ratio emerges to resolve parent-offspring conflict in social hymenopterans. We suggest
339 that genetic control of sex ratio should be investigated in other social insects,
340 particularly in those that do not conform to theoretical predictions.

341 **Materials and Methods**

342 *Sampling and Field Observations*

343 We sampled a mixed population of *F. glacialis* and *F. podzolica* 50 km west of
344 Whitehorse, Yukon Territory, Canada in July 2016. We removed the top 5-10 cm of soil
345 from each nest mound and assessed the presence and sex of winged sexuals. When
346 we observed strongly biased sex ratios (i.e., of the first 10 sexuals examined, at least
347 nine were of the same sex), we sampled at least eight workers and up to five males. We
348 did not sample gynes. In total, we sampled 71 *F. glacialis* colonies, of which 34 were
349 male-producing and 34 gyne-producing. The remaining three sampled colonies
350 contained no *F. glacialis* sexuals; two of the three also contained workers of the socially
351 parasitic species *Formica aserva*. We estimate that at least 80% of colonies with
352 winged sexuals exhibited a biased sex ratio.

353 *Whole-genome sequencing*

354 We sequenced 138 genomes of workers from 71 *F. glacialis* colonies. We
355 extracted Genomic DNA using the Qiagen DNeasy insect tissue protocol and prepared
356 whole-genome DNA libraries using a low-volume Illumina Nextera protocol (62) with the
357 following modifications: 2 ng/ μ L input DNA concentration instead of 0.5 ng/ μ L,
358 tagmentation reaction in 5 μ L volume instead of 2.5 μ L, PCR using Q5 DNA polymerase
359 (New England Biolabs) instead of KAPA HiFi, 90 seconds extension time in the thermal

360 cycling program instead of 30 seconds, and a 0.6:1 ratio of magnetic beads to sample in
361 the magnetic bead clean-up, instead of 1:1 ratio. The libraries were sequenced on an
362 Illumina HiSeq X-Ten by Novogene, Inc., using 150 bp paired-end reads.

363 *Variant calling*

364 We merged overlapping paired-end reads with PEAR v0.9.10 (63), aligned the
365 reads to the *F. selysi* reference genome (29) using BWA-MEM v0.7.17 (64), and
366 removed PCR duplicates with Samtools v1.8 (65). We called variants using Samtools
367 mpileup v1.8 (66) and filtered the genotypes for missing data (20% per locus, --max-
368 missing 0.8), minor allele count (--mac 2), and minimum depth (--minDP 1) with
369 VCFtools v0.1.13 (67).

370 *Population genetic analyses*

371 We identified regions significantly associated with colony sex ratio in 71 *F.*
372 *glacialis* colonies (n=138 individuals) by performing a Genome-Wide Association Study
373 (GWAS) using a univariate linear mixed model implemented in GEMMA v0.94 (68),
374 using a genetic similarity matrix to control for population structure as a random effect.
375 Three colonies without a sex ratio phenotype were assigned an "NA" phenotype. Upon
376 detecting a large region of chromosome 3 significantly associated with sex ratio, we
377 performed a principal component analysis (PCA) using Plink v1.90b3.38 (69) on
378 variants on this chromosome. We calculated Weir and Cockerham's F_{ST} (70) in 10 kbp
379 windows between the three genotype clusters identified in the PCA using VCFtools
380 v0.1.13 (67).

381 *Comparisons with sister species F. podzolica*

382 We examined the underlying genetics of split sex ratio in the sister species, *F.*
383 *podzolica*, as well. We sampled 12 colonies (5 male-producing, 7 gyne-producing) from
384 the same Yukon locality, and sequenced the genomes of 22 workers and called variants
385 using the same methods as for *F. glacialis*. We identified genetic clusters based on
386 variants on chromosome 3 and calculated F_{ST} between genetic clusters, again using the
387 methods described above for *F. glacialis*. We identified SNPs with alleles specific to the
388 Smd haplotypes of both species by comparing allele frequencies in four groups: *F.*
389 *glacialis* Sma/Sma, *F. glacialis* Sma/Smd, *F. podzolica* Sma/Sma, and *F. podzolica*
390 Sma/Smd. Loci with putative Smd-specific alleles shared between both species were

391 defined as those which have allele frequency between 0.4 and 0.6 in both of the
392 Sma/Smd groups, and allele frequency >0.95 or <0.05 in both of the Sma/Sma groups.
393 We plotted the frequency of SNPs meeting these criteria in 10 kbp windows along
394 chromosome 3.

395 *Social Organization*

396 We sampled 8 workers from 19 additional *F. glacialis* colonies in Alaska, British
397 Columbia, and Alberta, where no winged sexuals were visible at the time of collection.
398 We genotyped 145 of these workers using the double-digest RAD sequencing protocol
399 of Brelsford et al. 2016 (71), with restriction enzymes SbfI and MseI. RAD libraries were
400 sequenced on the Illumina HiSeq 4000 platform by the QB3 Genomics core facility of
401 University of California Berkeley, with 100bp paired-end reads. We aligned reads and
402 called variants using the procedures described above for whole-genome data, but
403 omitting the removal of PCR duplicates. Raw variants were filtered using VCFtools
404 v0.1.13 (67), retaining genotypes with sequence depth of at least 7 and variants with
405 genotype calls in at least 80% of samples.

406 To assess social organization of the 19 colonies, we calculated the number of
407 loci exhibiting opposing homozygosity within each colony, i.e., at least one worker
408 homozygous for the reference allele and one worker homozygous for the alternate
409 allele. In haplodiploid organisms, a male transmits the same allele to all of his offspring,
410 so in a group of full siblings, opposing heterozygosity is expected to be absent except in
411 the cases of genotyping errors or de novo mutations. Colonies with multiple queens, or
412 with a multiply mated queen, are expected to have a higher number of loci with
413 opposing heterozygosity.

414 We conducted a genome-wide association study for variants associated with
415 colony-level opposing homozygosity using a linear mixed model implemented in
416 Gemma v0.94 (68), which uses a relatedness matrix to control for non-independence of
417 samples. Finally, we carried out a principal component analysis of variants on
418 chromosome 3 on a merged dataset of whole-genome and ddRAD *F. glacialis*
419 genotypes. We generated a list of variants on chromosome 3 present in both the whole-
420 genome and ddRAD filtered VCF files, extracted those variants from both datasets, and

421 generated a merged VCF using VCFtools v0.1.13 (67). We used Plink v1.90b3.38 (69)
422 to carry out a principal component analysis on the resulting merged genotypes.

423 *Colony genotype distributions*

424 We designed a targeted PCR-RFLP assay for a trans-species SNP tagging
425 alternative chromosome 3 haplotypes in both *F. glacialis* and *F. podzolica*. We designed
426 primers (CTGGAACAACGGATCCTCA and TTCGCGATTCTGAATTTCTC) to amplify a
427 338 bp fragment, which, when digested with the restriction enzyme MluCI, produces
428 fragments of 325 and 13 bp for the haplotype associated with gyne production and 223,
429 102, and 13 bp for the haplotype associated with male production. We used this assay
430 to genotype 6 additional workers and any available males for all colonies of both
431 species.

432 We designed a second PCR-RFLP assay for a trans-species SNP broadly
433 conserved across *Formica* that differs between Sm and Sp alleles of the gene *knockout*.
434 Primers GGTGGYTCTTTCAACGACG and GCCATGTTCACCTCCACCA amplify a 230
435 bp fragment, which when digested with the restriction enzyme HinfI produces fragments
436 of 132 and 98 bp for the Sm allele and 230 for the Sp allele. We used this assay to
437 genotype 6 additional workers and any available males from three *F. glacialis* colonies
438 where initial whole-genome sequencing of two workers identified the presence of the Sp
439 allele at *knockout*. For both PCR-RFLP assays, we visualized the distinct banding
440 patterns with 2% agarose gel electrophoresis.

441 We constructed bar plots of supergene genotype frequency by colony based on
442 whole-genome sequencing and PCR-RFLP genotyping for the Yukon population, and
443 based on ddRAD for Alaska, Alberta, and British Columbia populations.

444

445 **Competing interest information for all authors**

446 The authors declare no competing interests.

447 **Data Sharing**

448 Raw sequences will be uploaded to NCBI SRA (accession numbers to be determined
449 pending acceptance). Colony metadata including locality, observed sex ratio, and
450 inferred social structure is included in Dataset S1.

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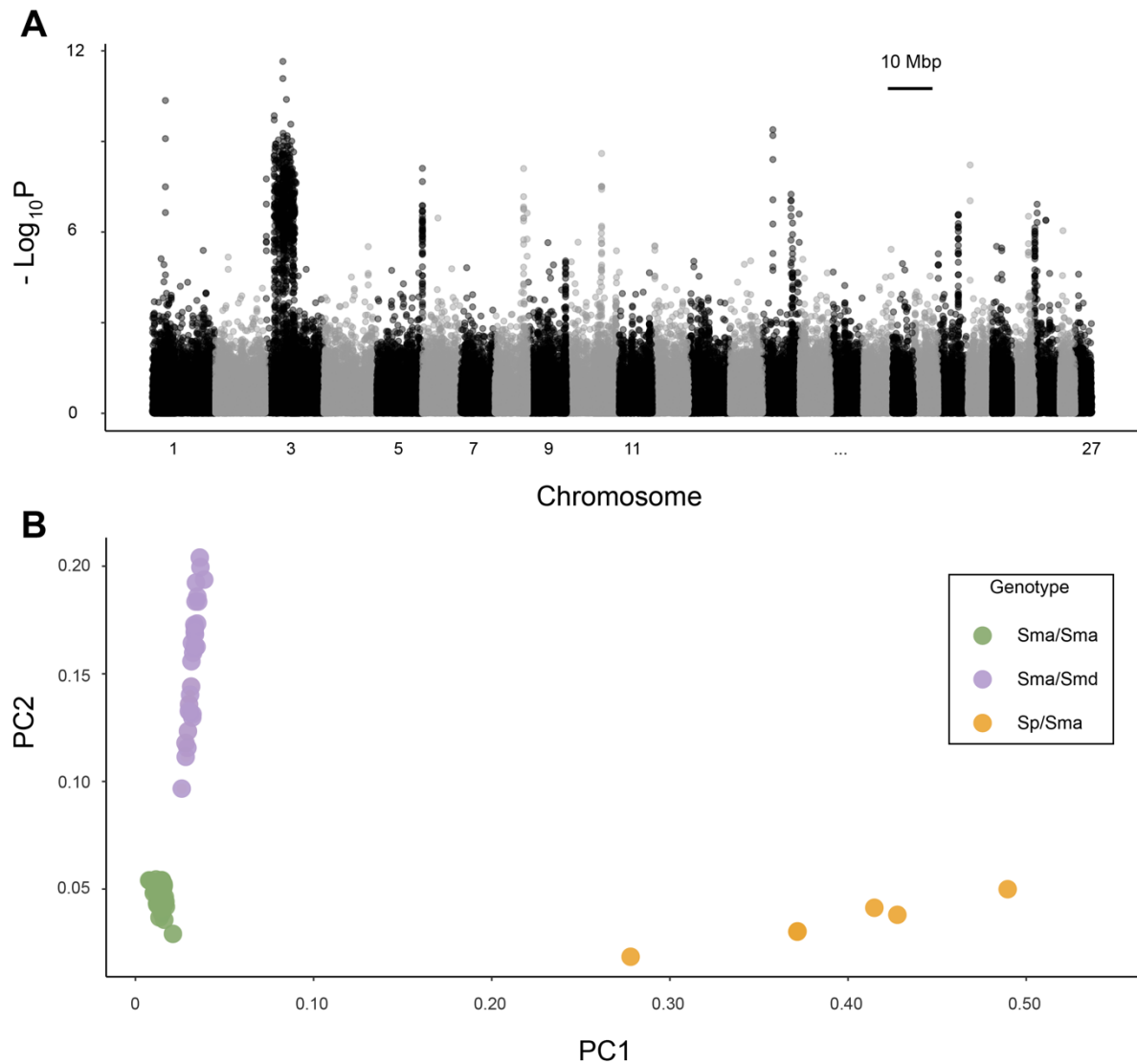
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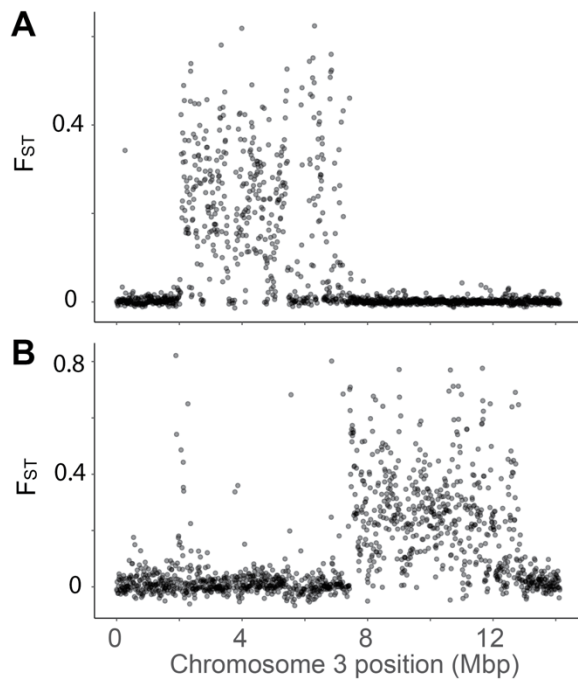
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622 **Figures**



623
624 Figure 1. (A) A genome-wide association study reveals a large region on chromosome 3
625 significantly associated with colony sex ratio in *F. glacialis*. (B) A principal component analysis
626 (PCA) of variants on chromosome 3 identifies three clusters corresponding to three genotypes,
627 Sma/Sma (green), Sma/Smd (purple), and Sp/Sma (yellow).

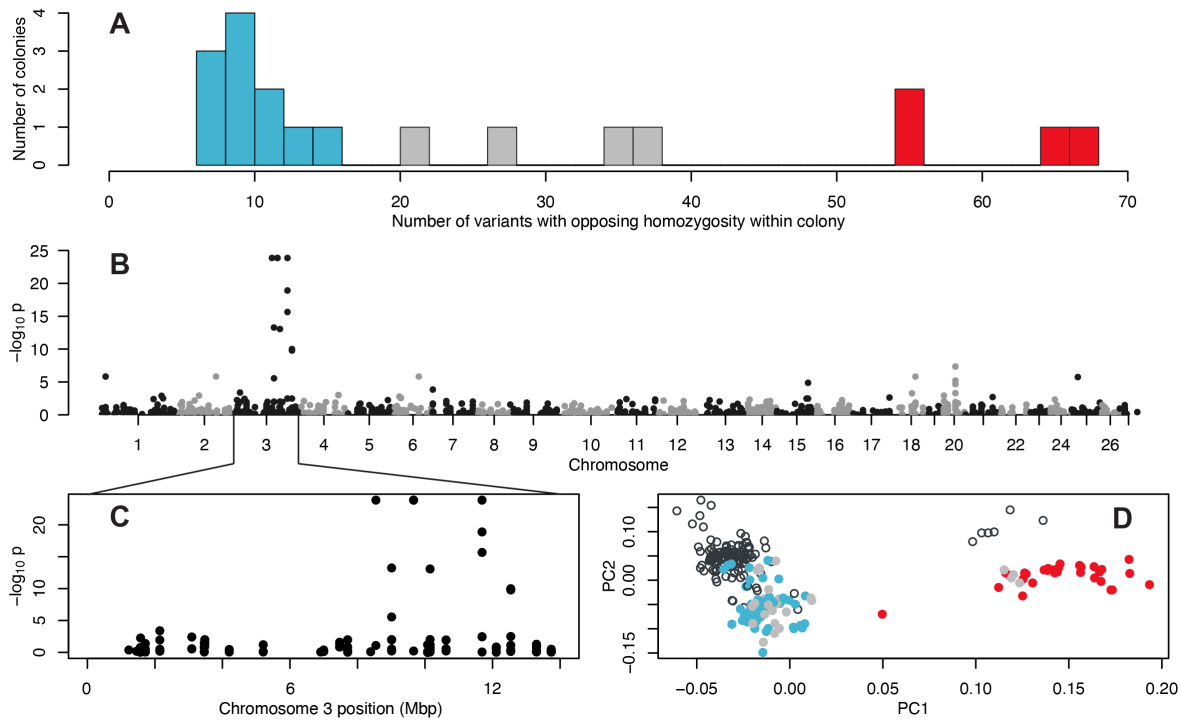
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630 Figure 2. Genetic differentiation between individuals with alternative genotypes on chromosome
631 3 reveals two adjacent supergenes in *F. glacialis*. (A) Sma/Sma workers and Sma/Smd workers
632 exhibit elevated F_{ST} between 2 Mbp and 7.5 Mbp. (B) Sma/Sma workers and Sp/Sma workers
633 exhibit elevated F_{ST} between 7.5 Mbp and 12.5 Mbp, with a small peak at 2 Mbp. Points
634 represent 10 kbp windows.

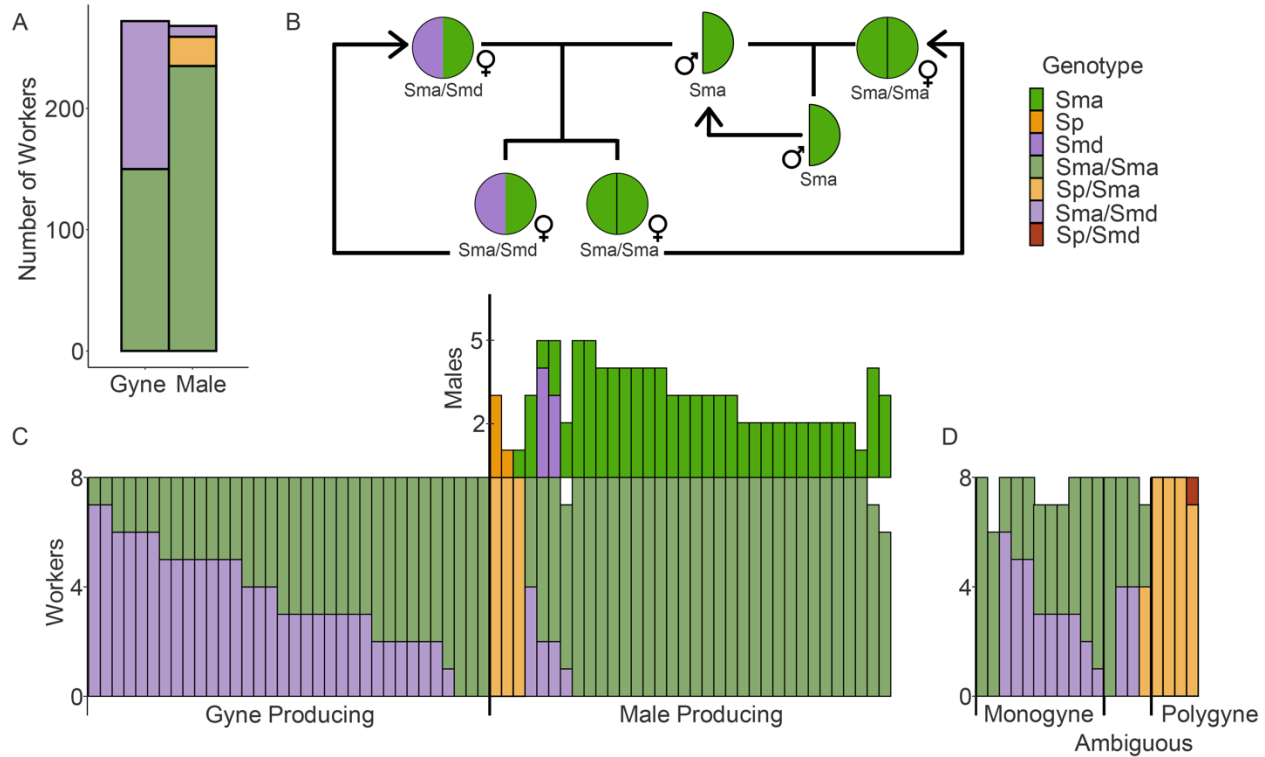
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637 Figure 3. The Sp supergene haplotype is associated with polygyne social structure in *F. glacialis*
638 from Alaska, British Columbia, and Alberta, based on RADseq genotyping of 7-8 workers from
639 each of 19 colonies. (A) Opposing homozygosity varies among colonies. Putative monogyne
640 colonies are colored blue, putative polygyne colonies are colored red, and undetermined are
641 colored gray. (B) GWAS revealed multiple SNPs associated with colony-level opposing
642 homozygosity on chromosome 3. (C) Significantly associated SNPs occur within the 7.5-12.5
643 Mbp region, also identified in the Yukon population and shown in Fig. 2b. (D) A PCA of variants
644 in both the RADseq (filled circles) and whole genome datasets (open circles) shows that the Sp
645 haplotype identified in the Yukon population clusters with the haplotype associated with
646 polygyne social structure in other populations. The majority of individuals from the Yukon cluster
647 with workers from monogyne colonies in other populations.

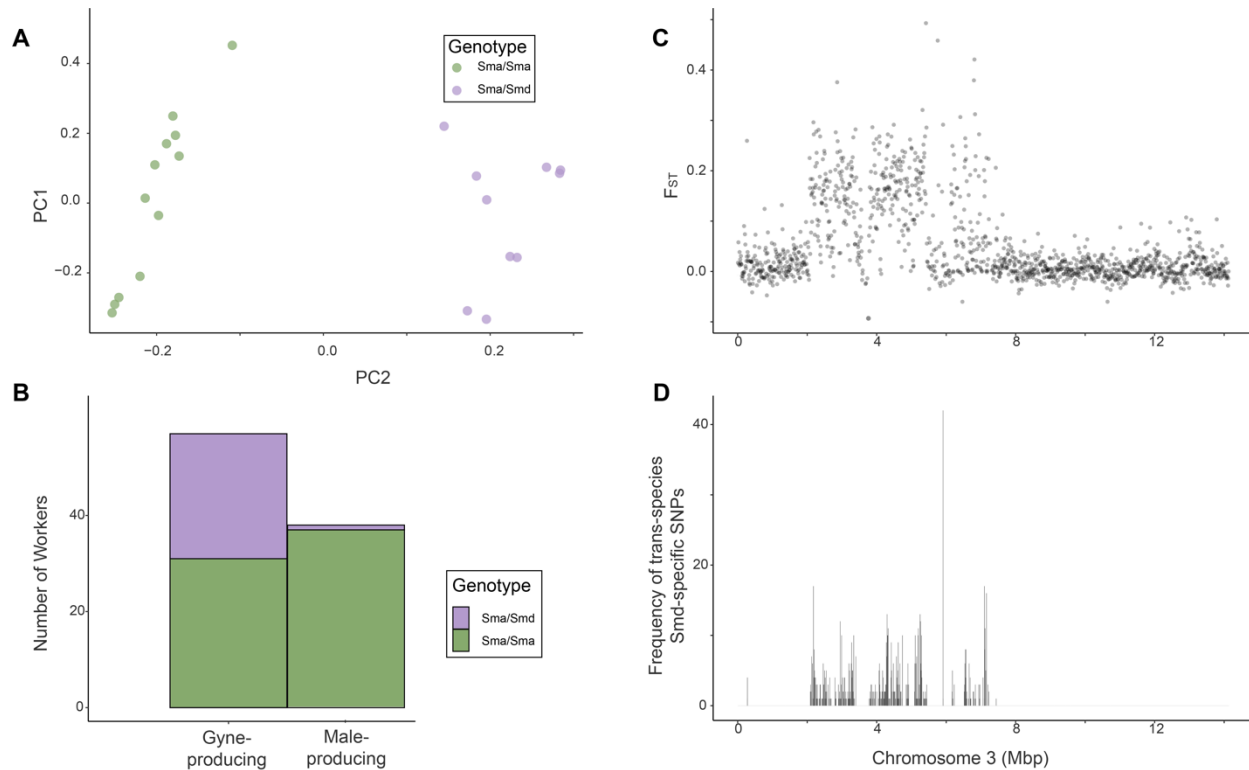
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650 Figure 4. (A) The Sma/Smd genotype in *F. glacialis* occurs in approximately half of workers from
 651 gyne-producing colonies, and is rare in workers from male-producing colonies. (B) We propose
 652 a model of Mendelian inheritance for maintenance of this supergene system in a largely
 653 monogyne population. Heterozygous queens (Sma/Smd) mated with a Sma male produce
 654 exclusively female offspring with Sma/Smd and Sma/Sma genotypes. Gynes with the
 655 heterozygous genotype become gyne-producers, while homozygotes become male-producers.
 656 (C) Gyne-producing colonies usually harbor a mix of Sma/Sma and Sma/Smd workers (31/34
 657 colonies). Male-producing colonies usually contain exclusively Sma/Sma workers and produce
 658 Sma males (27/34 colonies). Three additional male-producing colonies contain exclusively
 659 Sp/Sma workers and either Sp or Sma males. We did not detect Sma/Smd workers in three
 660 gyne-producing colonies, while we found at least one Sma/Smd worker in four male-producing
 661 colonies, indicating that the genetic basis of split sex ratio is imperfect in this system. (D)
 662 Among monogyne colonies from the broader geographic sample, two contain exclusively
 663 Sma/Sma workers, while nine contain Sma/Sma and Sma/Smd workers. All workers from
 664 polygyne colonies carry one Sp haplotype. The majority are Sp/Sma, and we detected one
 665 Sp/Smd worker.

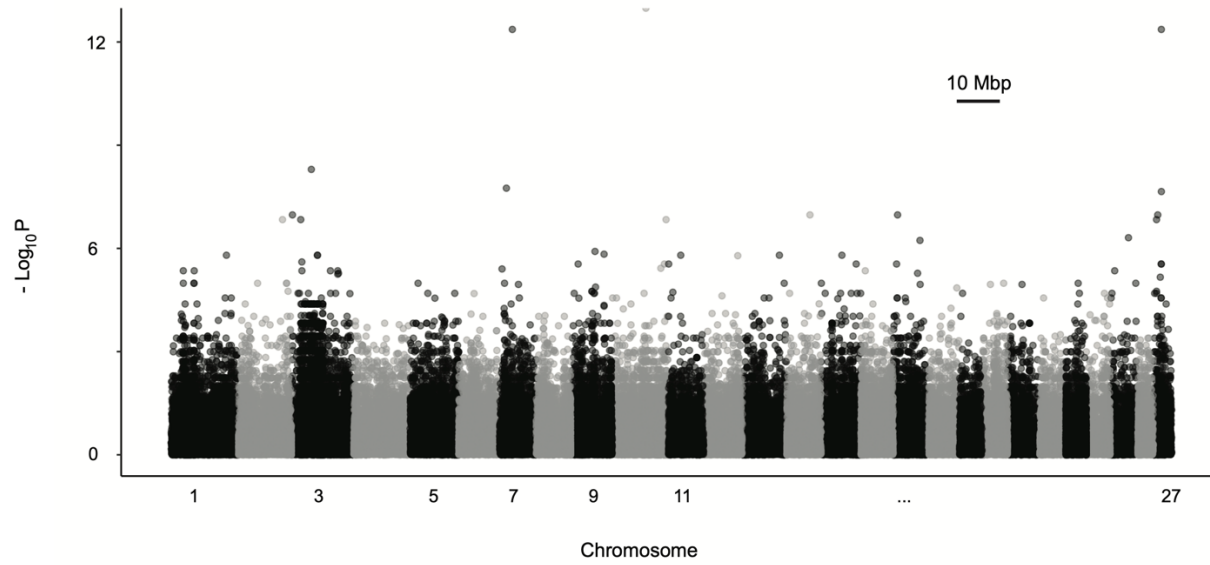
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668 Figure 5. Alternative genotypes matching those detected in *F. glacialis* are also associated with
669 colony sex ratio in *Formica podzolica*. (A) A PCA of genetic markers on chromosome 3 reveals
670 two genotype clusters, Sma/Sma (green) and Sma/Smd (purple). (B) Among workers from
671 gyne-producing colonies, about half carry the Sma/Sma genotype and half carry the Sma/Smd
672 genotype. In contrast, workers from male-producing colonies are almost exclusively Sma/Sma.
673 (C) In *F. podzolica*, the region of elevated F_{ST} between Sma/Sma and Sma/Smd workers
674 spanned 2-7.5 Mbp along chromosome 3. Points represent 10 kbp windows. (D) The *F.*
675 *glacialis* and *F. podzolica* sex ratio supergenes contain shared SNPs between the putative
676 homologous haplotypes.

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679 **Fig. S1.** Manhattan plot of GWAS results in *Formica podzolica* shows no clear association between
680 colony sex ratio and chromosome 3 supergene region based on 22 sequenced workers from seven gyne-
681 producing and five male-producing colonies.

682