Lethal epistasis maintains strong linkage disequilibrium between unlinked supergenes

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Abstract

Linkage disequilibrium (LD) between adaptive gene combinations is typically maintained through physical linkage and suppressed recombination. Although epistatic interactions can maintain LD between unlinked loci, this mechanism is rarely documented in nature. Supergenes, regions of suppressed recombination containing tightly linked loci, typically exemplify the first mechanism, in which genomic rearrangements lock together coadapted alleles. Here, we demonstrate a rare case of epistasis-driven LD between two supergenes on different chromosomes in the European ant *Formica cinerea*: one controlling colony queen number (chromosome 3) and another determining sexual body size (chromosome 9). We show that these supergenes assort independently according to Mendelian expectations during meiosis, yet exhibit high LD between the multi-queen haplotype P_2 and the miniaturizing haplotype 9r, with small queens and males occurring exclusively in multi-queen colonies. Mismatched genotype combinations (P_2 without 9r and vice versa) are severely underrepresented among all adult castes, with mismatched males experiencing complete mortality. We show that this pattern cannot be explained by meiotic drive or maternal-effect killing, indicating strong postzygotic epistatic selection maintaining the observed LD. The fitness costs of these epistatic interactions are substantial but critically depend on mating combinations: heterozygous small queens achieve 1.5-2.5 times higher fitness (based on offspring genotype viability) when mated to small males (P_2-9r) compared to large males. Our findings provide empirical evidence for epistatic interactions between unlinked supergenes maintaining LD in the absence of physical linkage.

Introduction

Understanding the link between genotype, phenotype, and fitness is a key step toward unveiling the genetic mechanisms that drive evolutionary adaptation within species. In 1930, Fisher conceptualized adaptation as populations evolving toward a single fitness optimum through

phenotypic changes caused by mutations, assuming a relatively smooth fitness landscape where mutations have predictable, consistent effects on fitness (Fisher 1930; reviewed by Orr 2005). In this regard, a mutation could be classified as beneficial or deleterious based on its intrinsic properties. Around the same time, Wright suggested a more complex view of the adaptive process, emphasizing the role of epistasis in shaping fitness (Wright 1931; reviewed by Bank 2022). Epistasis, defined as interactions between genes, creates rugged fitness landscapes with multiple adaptive peaks that correspond to different advantageous genetic combinations within a population.

In scenarios involving epistasis, the effect of a mutation on fitness is not intrinsic but depends on the genetic background in which it occurs. For example, an identical mutation can cause degenerative symptoms in one person but not in another (healthy carrier), depending on the genetic context (reviewed by Kammenga 2017). Epistatic interactions that influence survival are expected to produce some degree of linkage disequilibrium (LD), since individuals with high-fitness combinations of alleles will be more common than those with other combinations. Under some conditions, this is expected to select for recombination modifiers that further increase LD (Charlesworth 1993; Feldman et al. 1997). Broadening our understanding of the conditions that result in fitness-based LD *versus* the appearance of recombination modifiers in empirical systems is a much-needed step in exploring the evolutionary consequences of epistasis.

In cases with fitness-based LD, selection against maladaptive gene combinations can maintain linkage disequilibrium between beneficial allelic combinations, even without physical linkage. This creates functional associations that persist despite ongoing recombination. Selection maintaining epistatic interactions between physically unlinked loci has been documented across diverse organisms throughout the tree of life, with varying evolutionary consequences. For example, in the protozoan parasite *Toxoplasma gondii*, epistatic interactions among seven loci influence virulence traits (Lehmann et al. 2004). In wheat, epistasis between loci on different chromosomes may reduce the effective population size of crosses carrying incompatible allelic combinations (Tessele et al. 2025). In stick insects, epistatic interactions shape color morphs that experience differential survival (Nosil et al. 2020). However, if deleterious combinations of alleles are lethal, this imposes substantial fitness costs through the loss of nonviable offspring.

This high cost can be mitigated through the evolution of recombination suppression between interacting loci, which can be achieved through genome structural rearrangements, including inversions (if the loci are already on the same chromosome), translocations, or chromosomal fusions (Charlesworth & Charlesworth 1973). Classical models of sex chromosome evolution exemplify this mechanism. Suppressed recombination is favored when a sex-determining gene is linked to sexually antagonistic alleles, variants that are advantageous in one sex but detrimental in the other (Charlesworth and Charlesworth 1978; Charlesworth et al. 2005). This suppression of recombination can continue to expand in a stepwise fashion. Several empirical cases demonstrate how regions of suppressed recombination in ancestral sex chromosomes expand through chromosomal fusions with autosomes, creating neo-sex chromosomes. Examples include threespine sticklebacks (Kitano et al. 2009), various bird species (Gan et al. 2019; Huang et al. 2022, Muirhead et al. 2025), and butterflies (Mongue et al. 2017), although many of these examples do not clearly document a role of fitness epistasis in the evolution of neo-sex chromosomes.

Supergenes, clusters of tightly linked functional genetic elements that control discrete, complex phenotypes, typically exemplify the first mechanism, where genomic rearrangements, often inversions, lock together coadapted combinations of loci as evidence of past epistatic events (Gutiérrez-Valencia et al. 2021). However, epistatic interactions can still be detected within supergenes, as demonstrated in the land snail *Cepaea nemoralis*, where, despite suppressed

recombination, some individuals exhibit intermediate coloration and banding patterns that deviate from the expected discrete morph classes (Gonzalez et al. 2019).
At a larger scale, entire inversions can interact epistatically with each other, creating complex multi-locus systems where different chromosomal rearrangements function as interacting units. This phenomenon is observed in *Drosophila pseudoobscura*, where epistasis acts between three non-overlapping inversions on the same chromosome that together form a sex-ratio supergene (Fuller et al. 2020).

Despite the growing body of literature describing new supergenes in a variety of organisms (reviewed by Purcell & Brelsford 2025, and Gutiérrez-Valencia et al. 2021), we still know little about the role of epistasis in generating, maintaining, or expanding these large regions of suppressed recombination. Investigating epistatic interactions between well-established supergenes and genetic polymorphisms in other parts of the genome could help to bridge this knowledge gap. Here, we leverage a unique natural system where two supergenes co-occur within a single species (Scarparo et al. 2023), providing an opportunity to investigate inter-supergene epistatic interactions.

In several ant genera (*Solenopsis* (Wang et al. 2013), *Formica* (Purcell et al. 2014), *Pogonomyrmex* (Errbii et al. 2024), *Cataglyphis* (Lajmi et al. 2024), *Leptothorax* (Braim 2015), *Myrmica* (Sigeman et al. 2025a), and *Myrmecina* (Mona et al. 2025)), supergenes have convergently evolved to control colony social form and other fitness-related traits (Purcell and Brelsford 2025, De Gasperin et al. 2024; Lawson et al. 2012). In *Formica*, an ancient supergene, dating back approximately 30 million years, is widespread within the genus and spans much of chromosome 3 (Brelsford et al. 2020; Purcell et al. 2021). In several *Formica* species, this supergene has been consistently linked to colony queen number (Purcell et al. 2014; McGuire et al. 2022; Pierce et al. 2022; Purcell et al. 2025), although exceptions have been observed (Scarparo et al. 2024; Lagunas-Robles et al. 2025; Sigeman et al. 2025b).

Recently, Scarparo et al. (2023) described a novel supergene system in the European *Formica cinerea*. This species carries four supergene haplotypes (M_A, M_D, P_1, and P_2) on chromosome 3, as well as a second supergene on chromosome 9 with two alternative haplotypes (9a and 9r). Beyond influencing colony queen number, this complex system is also associated with colony split sex ratio, similar to the results reported in several North American *Formica* species (Lagunas-Robles et al. 2021; Lagunas-Robles et al. 2025), and with alate body size. In *F. cinerea*, Scarparo et al. (2023) identified three distinct queen morphs (Table 1): (1) monogyne macrogyne queens, carrying only M haplotypes on chromosome 3 and homozygous 9a9a on chromosome 9; (2) polygyne macrogyne queens, approximately 5% smaller than their monogyne counterparts, carrying at least one P_1 haplotype (but not P_2) on chromosome 3 and 9a9a on chromosome 9; and (3) polygyne microgyne queens, up to 20% smaller than macrogyne queens, carrying at least one P_2 haplotype on chromosome 3 and at least one 9r on chromosome 9. Males carrying the P_2–9r combination are, on average, 9% smaller than males without 9r (Table 1). In this initial study, the P_2 haplotype showed strong LD with the 9r haplotype (Scarparo et al. 2023).

Scarparo et al. (2023) proposed that small queens are strongly disadvantaged in a monogyne background, where they would be the sole reproductive individual, and that strong selection, therefore, could have favored linkage between the polygyne-associated haplotype P_2 and the miniaturizing haplotype 9r.

Here, we investigate the nature of the observed LD between the two *F. cinerea* supergenes and ask whether the fitness effects of one supergene depend on the genetic state of another. We considered two possible mechanisms that could explain strong LD between P_2 and 9r: physical linkage (e.g., chromosomal fusion or reciprocal translocation) that prevents recombination, or epistatic selection against mismatched genotypes (i.e., individuals carrying P_2 without 9r, or 9r

without P_2) that maintains LD despite recombination. The occasional occurrence of mismatched genotypes suggests that individuals with mismatches have reduced survival, but that the genotypic mismatch is not completely lethal. This opens the possibility that genotype combinations between chromosomes 3 and 9 generate a complex fitness landscape with differential survival of various genotype combinations. To distinguish between these mechanisms, we employ two complementary approaches. First, we analyze genotype frequencies in eggs to test for independent assortment, which would occur under epistatic selection but not under physical linkage. We also investigate possible gene drive mechanisms that could skew the expected Mendelian ratios, explaining the paucity of mismatched adult ants in our population sample. Second, we reanalyze genotype frequencies across adult morphological castes (workers, gynes, and males) from our previous dataset (Scarparo et al. 2023) to identify potential epistatic effects manifesting as deviations from expected frequencies.

Materials and Methods

Sample collection

Formica cinerea queens were collected in the Aosta Valley and Piedmont regions in Italy between late June and early July over multiple years from 2019 to 2022. Queens were either collected directly from colonies along with several workers (classified as mature queens) or captured after mating flights while clinging to vegetation or walking on the ground in search of suitable nest sites (classified as newly mated queens).

After collection, each queen was kept individually in a glass tube. Newly mated queens were provided only water, while mature queens were given a sugar-water solution and accompanied by 3–4 workers from their original colony. Brood production was monitored every other day, and eggs or larvae were collected once at least 10 brood items were present (this was repeated if queens continued to lay eggs). Queens were kept alive for an average of two months before being preserved in 100% ethanol for genetic analysis.

Colony fragments with gynes (unmated queens), males and workers were collected from the same locations, during the same years and seasons, as the queens. These samples were previously sequenced and discussed in Scarparo et al. (2023) to describe the supergene system in *F. cinerea*.

PCR-RLFP assays

We developed five PCR–restriction fragment length polymorphism (PCR-RFLP) assays to distinguish the four supergene haplotypes on chromosome 3, and two additional assays to differentiate the two haplotypes on chromosome 9 in *F. cinerea*. Each assay used two pairs of primers (see Table S1, columns “External primer sequences” and “Internal primer sequences”) to perform nested PCR, which is particularly effective when working with low DNA quantities, such as those extracted from single eggs or spermathecae. Each primer set was paired with a specific restriction enzyme targeting a diagnostic single-nucleotide polymorphism (SNP), which allowed us to distinguish haplotypes based on the presence or absence of restriction cuts in a short amplified fragment. For details on the DNA extraction and genotyping protocols, primer sequences, and corresponding enzymes, see the supplementary files and Tables S1-S2.

Meiotic Drive and independent assortment of Chr 3 and 9 in eggs

In a previous study, Scarparo et al. (2023) observed strong association between the P_2 and 9r haplotypes. However, the nature of this association remains unclear. If it is due to physical linkage, maternal P_2 and maternal 9r should be co-transmitted nearly 100% of the time in offspring of queens heterozygous for both supergene haplotypes, with only rare exceptions caused by recombination events. Alternatively, if the association reflects selection against “mismatched” combinations (epistasis) later in development, we would expect the four possible maternal haplotype combinations (P_2–9r, P_2–9a, X–9r, X–9a; where X denotes a maternal non-P_2 haplotype) to occur at equal frequencies (25% each) under independent assortment, assuming no meiotic drive. For simplicity, given that our focus for this study is the P_2 and 9r haplotypes, we

refer in the following analyses to all non-P_2 haplotypes collectively as "X" (including M_A, M_D, and P_1), recognizing that this encompasses both monogyne haplotypes and the alternative polygyne P_1 haplotype.
Because supergene haplotypes can act as selfish genetic elements that distort transmission (e.g. Avril et al. 2020), we first tested whether P_2 on chromosome 3, 9r on chromosome 9, or both deviate from Mendelian segregation by genotyping 127 eggs from 16 P_2-heterozygous queens and 164 eggs from 20 9a9r queens. We then examined the association between maternal haplotypes on chromosomes 3 and 9 by genotyping 100 eggs from 14 XP_2–9a9r queens. Since diploid eggs receive one chromosome from each parent, maternal haplotypes transmitted to eggs were inferred by subtracting the known paternal contribution (determined by genotyping sperm from the spermatheca) from each egg's diploid genotype. For example, if an XP_2 queen mated with an X male and produced 50% XX eggs and 50% XP_2 eggs, the maternal haplotype transmission would be 50% X and 50% P_2.

Maternal-effect killing
The consistent presence of the P haplotype across all individuals in polygyne colonies of *F. selysi* has been attributed to a maternal-effect killer mechanism (Avril et al. 2020). In *F. selysi*, heterozygous MP queens mated to M males lay 50% MM eggs and 50% MP eggs, but only MP eggs successfully hatch. This gene drive mechanism has been proposed to function as a toxin-antidote system, where eggs that do not inherit the P-associated antidote (i.e., MM eggs laid by MP queens) die due to the maternally transmitted toxin. Given that *F. selysi* and *F. cinerea* share homologous supergenes on chromosome 3 and are phylogenetically close (Purcell et al. 2021), we investigated whether haplotype frequencies could be distorted by a similar maternal-effect killer in *F. cinerea*.
We calculated genotype frequencies for chromosomes 3 and 9 in eggs and larvae whose mothers are XP_2 (n=13 for eggs and n=12 for larvae) or 9a9r (n=14 for eggs and larvae) and mated with X-9a males (M_A-9a or P_1-9a). We analyzed whether the proportion of XP_2 and 9a9r offspring differed between eggs and larvae.
Moreover, we investigated whether transmission of the four possible maternal haplotype combinations (P_2–9r, P_2–9a, X–9r, X–9a) from double heterozygous XP_2-9a9r queens occurred at equal frequencies in eggs and larvae (at the expected rate of 25% for each haplotype combination), or whether some combinations were underrepresented in larvae compared to eggs, indicating early mortality.

Epistasis in adults
To test for signatures of epistasis that might have led to LD between P_2 and 9r, we re-analyzed the dataset from Scarparo et al. (2023). In that study, we collected *F. cinerea* colony fragments without prior knowledge of their genotypes and genotyped up to five workers, eight gynes, and eight males per colony using RAD-seq. Gynes and males were collected before mating flights, often during the pupal stage, and genotyped after eclosion. Importantly, the genotypes of the reproductive queens were not directly known. This dataset includes 699 workers, 190 gynes, 384 males, and 123 mated queens. We added an additional 52 mated queens collected in 2022 and 123 spermathecae that were not included in the previous study (Table S2).
For the present study, we compared observed and expected genotype combinations separately for workers, gynes, mated queens, spermathecae, and males to assess whether certain combinations occurred more or less frequently than expected.
We further investigated whether some types of mismatches are more common than others (P_2-9a vs X-9r), and whether workers show higher mismatch frequencies than the reproductive caste. Finally, we tested whether mismatch frequencies differ before and after mating in queens to

assess whether observed genotype frequencies result from extrinsic selection (e.g., mating failure or unsuccessful colony founding) or from intrinsic genetic incompatibilities between haplotypes.

Statistical analyses

To test whether haplotype transmission is biased by meiotic drive, we first analyzed eggs laid by heterozygous XP_2 queens (chromosome 3) and 9a9r queens (chromosome 9). For each queen, we calculated the proportion of eggs inheriting the maternal P_2 or 9r haplotype and fitted two generalized linear models (GLMs) with a binomial error distribution [using the glm function in R, lme4 package (Bates et al. 2015)], one for chromosome 3 and one for chromosome 9. In each model, the response variable was the proportion of eggs inheriting the maternal P_2 (chromosome 3) or 9r (chromosome 9) haplotype, with male genotype (the queen's mate) included as a fixed factor. Models were weighted by the total number of eggs analyzed per queen.

To test whether P_2 and 9r haplotypes are transmitted independently and whether the mate genotype has an effect on their transmission, we modeled the maternal haplotype combination in eggs (categories P_2-9a, P_2-9r, X-9a, X-9r) as a multinomial response. Predictors included the mate genotype as a fixed effect and queen identity as a random intercept to account for repeated measures of eggs from the same queen. We excluded from this analysis double-mated queens. We used a Bayesian multinomial regression [brms, family = categorical, N = 145 eggs, 16 queens, 8000 post-warmup draws (Bürkner 2017)]. We fit models with both P_2-9a and P_2-9r as reference categories to facilitate interpretation, then used the hypothesis function to test whether transmission probabilities differed between mate genotypes.

To test whether P_2 and/or 9r act as maternal-effect killers to increase their representation in the next generation, we compared genotype frequencies in eggs and larvae produced by heterozygous XP_2 queens (chromosome 3) mated with X males and 9a9r queens (chromosome 9) mated with 9a males. For each queen, we calculated the proportion of XP_2 and 9a9r offspring in eggs and larvae and fitted two generalized linear mixed models (GLMMs) with binomial error distributions (using the glm function in R), one for chromosome 3 and one for chromosome 9. In each model, the response variable was the proportion of offspring inheriting the maternal P_2 (chromosome 3) or 9r (chromosome 9) haplotype, with developmental stage (eggs vs. larvae) included as a fixed factor and queen ID as a random effect. Models were weighted by the total number of eggs and larvae analyzed for each proportion.

Deviation of maternal haplotype combinations (P_2-9a, P_2-9r, X-9a, X-9r) from the expected 25% in larvae produced by double heterozygous XP_2-9a9r mothers was analyzed with a Bayesian multinomial regression (brms, family = categorical, N = 98 larvae, 14 queens, 8000 post-warmup draws) using the P_2-9a haplotype combination as the reference category. Predictors included mate genotype (X-9a and P_2-9r) as a fixed effect and queen identity as a random intercept. We then refit the model with identical settings but using P_2-9r as the reference category to facilitate interpretation of contrasts. We used the hypothesis function to test whether the transmission probability of each haplotype combination differed between mate genotypes.

We next tested whether certain genotype combinations across chromosomes were overrepresented among workers, gynes, mated queens or males. For each caste, we constructed contingency tables of genotype combinations and compared observed to expected counts under the assumption of independence between loci (i.e., no epistasis between the supergene on chromosome 3 and chromosome 9) using chi-square tests. Standardized residuals were calculated for each cell. For males, we only assessed haploid individuals, excluding two diploids.

Finally, we examined whether mismatched haplotype combinations (P_2–9a or X–9r) were distributed unevenly among castes. To test for asymmetry in the occurrence of the two mismatched classes, we additionally performed a binomial test to assess deviations from a 1:1 ratio between P_2–9a and X–9r genotypes across all 58 mismatched individuals. We then examined whether non-reproductive individuals (workers) carried more mismatched genotypes than reproductives. We categorized mismatched individuals into two groups: those carrying one P_2-9r combination (P_2P_2-9a9r and XP_2-9r9r genotypes) and those with a P_2 haplotype but no 9r and vice versa (XP_2-9a9a and XX-9a9r genotypes). We fit a GLM [glm function in R, lme4 package Bates et al. 2015)] with a binomial error distribution with mismatch type as the response

variable (0 = with P_2-9r combination, 1 = without P_2-9r combination) and included caste (reproductive vs. worker) as a fixed effect. Finally, we tested whether unmated gynes carried more mismatches than mated queens. We fit a GLM with a binomial error distribution with mismatch presence/absence as the response variable (0 = non-mismatched, 1 = mismatched) and included mating status (mated vs unmated) as a fixed effect.

Results

No evidence for meiotic drive for either of the two supergenes

We fit two binomial GLMs to test whether maternal haplotype transmission on chromosomes 3 and 9 deviated from Mendelian expectations (50%). In XP_2 queens, transmission of P_2 did not differ from 50% either when mated with X males (43.6% P_2; Wald test: z = –0.81, p = 0.43) or with P_2 males (58.6% P_2; z = 1.27, p = 0.20; Fig. 1A). Similarly, we found no effect of mate genotype on transmission of 9r: 9a9r queens mated with 9a males (51.7% 9r; z = 0.36, p = 0.71) or 9r males (48.2% 9r; z = –0.26, p = 0.79) did not deviate from equal transmission (Fig. 1B).

Evidence of independent assortment between the supergenes

Across the 16 XP_2–9a9r queens, the four maternal haplotype combinations occurred at similar frequencies in eggs, averaging across all queen and mate combinations (X–9r: 23.3%, 95% CrI = [9–42%]; X–9a:31%, 95% CrI = [11.5–49.8%]; P_2–9r: 26%, 95% CrI = [12–45.6%]; P_2–9a: 19.5%, 95% CrI = [9–35%]), with credible intervals overlapping the expected Mendelian frequency of 25% for all combinations. This reveals that haplotypes on chromosome 3 are transmitted independently from the haplotypes on chromosome 9 (Fig. 1C). Mate genotype (X-9a vs P_2-9r) did not significantly influence haplotype transmission frequencies (all mate effect credible intervals included zero: P_2-9r estimate = 0.02 [-1.35, 1.47]; X-9a estimate = 0.7 [-0.66, 2.13]; X-9r estimate = 0.21 [-1.49, 1.78]; P_2-9a estimate=-0.06 [-1.45, 1.3]).

No evidence that P_2 or 9r are maternal-effect killers

To investigate whether P_2 and/or 9r distort their transmission through maternal-effect killing, as discovered for the P haplotype in *F. selysi* (Avril et al. 2020), we fit generalized linear mixed models (GLMMs) to test for differences in the proportion of XP_2 or 9a9r brood between the egg and larval stages. ANOVA tests revealed no significant difference in the proportion of heterozygous brood for either P_2 (χ^2 = 3.08, df = 1, p = 0.08, Fig. 2C) or 9r (χ^2 = 0.05, df = 1, p = 0.84, Fig. 2B), suggesting that neither haplotype acts as a maternal killer.

To further test whether the transmission of the four haplotype combinations (P_2-9a, P_2-9r, X-9a, X-9r) differed from expected Mendelian ratios in larvae and whether their transmission differed compared to eggs, we fit a Bayesian multinomial regression model with queen as a random effect. Results for larvae mirrored those observed in eggs: posterior predictions showed similar transmission frequencies across all four haplotype combinations, averaging across all mates and queens (X-9r: 22.3%, 95% CrI = [12.8%, 33.2%]; X-9a: 24.9%, 95% CrI = [15.6%, 35.5%]; P_2-9r: 28.5%, 95% CrI = [18%, 40.4%]; P_2-9a: 24.3%, 95% CrI = [15,5%, 34.4%]), with all credible intervals overlapping the expected Mendelian frequency of 25%, indicating no evidence for transmission distortion (Fig. 2C). We did not detect any difference in the proportion of haplotype combinations between eggs and larvae (all caste effect credible intervals included zero: P_2-9r caste estimate 0.14 [-0.75, 1.03]; P_2-9a caste estimate= -0.17 [-1.07, 0.74]; X-9a caste estimate= 0.46 [-041, 1.33]; X-9r caste estimate= 0.28 [-0.61, 1.19]). Importantly, we detected MM larvae produced by MP_2 queens, indicating that the strong maternal effect killer discovered in *F. selysi* (Avril et al. 2020) is weak or absent in *F. cinerea* (Figure S2).

Specific mismatched genotypes are underrepresented in adults

We next tested whether genotypes on chromosomes 3 and 9 were associated in workers, gynes, mated queens, spermathecae and males. Fisher's exact tests revealed a highly significant association between the two supergenes in all three castes (workers: p < 0.0001; gynes: p < 0.0001; mated queens: p < 0.0001; spermatechae: p< 0.0001; males: p < 0.0001). Matched

genotype combinations (X-9a and P_2-9r) were consistently overrepresented relative to expectations, whereas most mismatched combinations were strongly underrepresented, with the exception of P_2P_2-9a9r and XP_2-9r9r genotypes. In diploids, certain mismatched combinations (P_2P_2–9a9a and XX–9r9r) were completely absent (Fig. 3A). Likewise, none of the 123 spermathecal content and the 384 haploid males analyzed carried a mismatched genotype, except for a single diploid male (P_2P_2–9a9r) (Fig. 3B, Table S2).
In total, we identified 58 mismatched individuals out of 1448. Despite their overall rarity, mismatches were asymmetrically distributed: the P_2–9a combination was significantly more common than X–9r ($p = 0.0003$), with 43 of the 58 mismatched individuals carrying at least one P_2–9a chromosome pair, including one diploid male (not shown in Fig. 3). Moreover, mismatched genotypes lacking P2-9r entirely (XP_2-9a9a and XX-9a9r) were far less common than genotypes with at least one P_2-9r combination (P_2P_2-9a9r and XP_2-9r9r). We therefore tested whether reproductive and non-reproductive individuals differed in the frequency of genotypes containing at least one P_2-9r combination. Among all diploid individuals with mismatched genotypes, reproductive individuals (gynes, queens, and one diploid male) were significantly more likely to carry at least one P_2-9r combination than workers (estimate = -2.09 ± 0.813 SE, $z = -2.568$, $p = 0.0102$). Only 2 out 19 (10.5%) of reproductive individuals carried mismatched genotypes lacking P_2-9r on both chromosome pairs (XP_2-9a9a and XX-9a9r genotypes), whereas 19 out of 39 (48.7%) of the mismatched workers lacked any P_2-9r combination. We did not find a significant difference in the frequency of mismatches between unmated gynes and mated queens (estimate = 0.2 ± 0.47 SE, $z = 0.43$, $p = 0.67$)

Discussion

The formation of maladaptive allele combinations is often resolved through recombination suppression within supergenes (Thompson & Jiggins 2014). Here, we provide empirical evidence of strong epistasis between two supergenes in *F. cinerea* that control social organization (monogyne vs. polygyne) and queen and male size (macrogynes/macraners vs. microgynes/micraners). These supergenes exhibit strong linkage disequilibrium (LD), with the polygyne-associated P_2 haplotype and the miniaturizing 9r haplotype co-occurring more frequently than expected (Scarparo et al. 2023). We demonstrate that antagonistic interactions impose severe viability costs on some mismatched genotype combinations, maintaining non-random haplotype associations across the genome without physical linkage and thereby coupling colony social form with queen body size.

LD between supergenes is maintained by asymmetric selection against mismatched genotypes

Our study demonstrates that the P_2 and 9r haplotypes assort independently during meiosis. Heterozygous XP_2–9a9r queens transmit all four possible haplotype combinations to eggs at the expected Mendelian ratio. This independent assortment rules out physical linkage as an explanation for the strong LD observed between supergenes. This finding is surprising because elevated LD typically results from reduced recombination between physically linked loci, which can occur due to close physical proximity on the same chromosomes or through chromosomal rearrangements, such as the inversions observed in many organisms [i.e. ruffs (Lamichhaney et al. 2016); fire ants (Yan et al. 2020); *F. selysi* (Brelsford et al. 2020); butterflies (Jay et al. 2021)], or through chromosomal fusion, as reported for mating-type loci in several fungi species (i.e. Duhamel et al. 2022; Lucotte et al. 2025).

Instead, in *F. cinerea* the LD between supergenes appears to be maintained by epistatic interactions between haplotypes. Genotype frequencies in the adult population deviated from expected frequencies. Certain genotype combinations show complete or near-complete lethality: all males with mismatched chromosomes die, while XX–9a9r and XP_2–9a9a diploids are severely underrepresented. Conversely, P_2P_2-9a9r and XP_2-9r9r genotypes are overrepresented, indicating that diploids carrying at least one P_2 haplotype generally require at least one 9r

haplotype to be viable. This pattern is consistent with recessive epistatic lethality, where at least one matched P_2-9r combination enables normal development. This constraint operates differently across castes: nearly 90% of reproductive individuals with mismatched genotypes carry at least one P_2-9r combination, whereas mismatched workers are evenly split between those with and without P_2-9r combinations. This suggests that complete decoupling of P_2 and 9r may bias diploid development toward the worker caste and reinforce P_2-9r coupling specifically in the reproductive caste.

Segregation distorters could account for deviations from expected genotype frequencies. We genotyped eggs and larvae to determine whether meiotic drive or a maternal-effect killer biased genotype ratios at early stages of development. Our data are not consistent with either segregation distortion mechanism. First, strong meiotic drive can be excluded: both P_2 and 9r are transmitted to eggs at approximately 50% frequency by heterozygous queens, with no evidence that mate genotype influences transmission (consistent with normal Mendelian segregation). Second, a strong maternal-effect killer mechanism, analogous to the system in *F. selysi* where MM eggs laid by MP queens fail to hatch (Avril et al. 2020), seems unlikely. Indeed, crosses between XP_2 queens and X males yield XP_2 genotypes at the expected ~50% frequency in both eggs and larvae, and 9a9r queens mated to 9a males similarly produce expected genotype ratios in larvae. These results are not consistent with maternal-effect killers targeting specific genotype combinations.
Assortative mating with regard to supergene haplotype has been reported in *F. selysi* (Avril et al. 2019) and for the Gp9 gene (later determined to be within the queen number supergene) in *S. invicta* (Ross & Keller 1995) and could theoretically produce some of the observed genotype frequency patterns in adults. However, assortative mating alone is insufficient to explain several key observations, including the complete absence of mismatched males in our population samples, and the specific asymmetries in which mismatch types survive (e.g., P_2-9a is more common than X-9r; see Fig. 3).
Taken together, our results point to fitness epistasis between the two supergenes as the primary mechanism shaping genotype frequencies in adults and elevated LD between chromosomes.

Postzygotic incompatibilities couple social organization and body size
The chromosome 3 supergene controls social organization, with different haplotypes associated with polygyny (P_1, P_2) or monogyny (M_A, M_D). The chromosome 9 supergene controls the body size of queens and males, with tiny individuals having at least one copy of the 9r haplotype. In natural populations, these traits show strong phenotypic associations: the P_2 and 9r haplotypes are found together far more frequently than expected by chance, with only polygyne colonies (and never monogyne colonies) containing tiny P_2-9r reproductive individuals (Scarparo et al. 2023). This phenotypic coupling could result from either low fitness in mismatched adults, or intrinsic lethality in individuals with mismatched haplotype combinations. If selection on adults drives the phenotypic association, mismatched males and gynes should develop normally and either fail to mate or be selected against post-mating (e.g., through unsuccessful colony foundation). In contrast, intrinsic lethality would prevent mismatched individuals from completing development. We tested these alternatives by comparing mismatch frequencies across developmental stages (eggs, larvae, and adults) and between reproductives before and after mating (males/gynes vs. sperm/queens).
Across the entire sample of eggs analyzed (n=571), mismatched genotypes occur at 17.5% frequency. This frequency increases to 44% when considering only eggs laid by queens heterozygous at one or both loci. Preliminary data from early-stage larvae from heterozygous queens show mismatched genotypes remain present at 48% frequency, suggesting that mismatch genotype-specific mortality occurs during later larval or pupal stages rather than before hatching. By the time we sampled adults, mismatched genotypes drop to just 5.2% in gynes and workers and to 0% in males in the whole population. All males were collected from colonies, some as pupae and some as newly eclosed adults, likely before mating (though we cannot exclude that some individuals mated with gynes within their natal colony). Similarly, of the 123

spermathecae we successfully dissected and genotyped, none carried mismatched sperm (Fig. 3B, Table S2). The complete absence of mismatched genotypes in haploids before (the males) and after (the sperm) mating strongly suggests that mismatched haplotype combinations are developmentally lethal rather than causing reduced fecundity or mating success. Indeed, if mismatched males developed normally but failed to mate, we would expect to observe them among the unmated male samples. Consistent with this interpretation, we detected no significant differences in mismatch frequency between unmated gynes and mated queens, further supporting developmental lethality over mating failure. Thus, intrinsic lethality rather than selection on adults plays a major role in maintaining both the genetic (P_2-9r) and phenotypic (polygyne–small body size) associations.

Mating incompatibilities reinforce lethal epistasis

The severity of epistatic interactions depends critically on mating combinations. Queens carrying P_2–9r haplotypes and heterozygous at one or both supergene regions experience dramatically different offspring viability depending on the mate genotype (Table 1). When mated with X–9a males, these queens are expected to produce many lethal genotype combinations, resulting in up to 75% offspring mortality. In contrast, mating with P_2–9r males yields predominantly viable offspring, even when some chromosome pairs carry mismatched allele combinations.

At the haplotype level, this translates to strong positive epistasis between P_2 and 9r, with recombinant (P_2–9a and X–9r) combinations imposing severe fitness costs, including complete lethality in haploid males. This pattern resembles Dobzhansky–Muller incompatibilities (reviewed by Orr & Turelli 2001), which typically describe genetic conflicts arising between alleles evolved in allopatric populations that secondarily come into contact. In *F. cinerea,* an analogous genetic mechanism could maintain LD between chromosomes 3 and 9, where co-evolved haplotypes (P_2–9r) are viable but recombinant combinations (P_2–9a, X–9r) cause lethality through negative epistasis.

This developmental pattern suggests that colonies containing heterozygous queens pay substantial energetic costs, as workers allocate resources in rearing larvae that ultimately die before completing development. Similar fitness costs from genetic incompatibilities occur in other systems. In polygyne colonies of *F. selysi*, eggs lacking the P haplotype fail to hatch (Avril et al. 2020). Beyond social insects, segregating inversions in yellow monkeyflowers (*Mimulus guttatus)* reduce pollen viability by approximately 30% (Lee et al. 2016), while white-throated sparrows carrying homozygous 2m/2m genotypes show reduced survival (Tuttle et al. 2016). These examples demonstrate that supergene systems commonly impose substantial fitness costs through the production of inviable or low-fitness genotype combinations.
In *F. cinerea*, despite these elevated fitness costs at both individual and colony levels, the P_2 and 9r haplotypes persist in the population at moderate frequencies (approximately 9%). If these incompatibilities created complete reproductive barriers between morphs, we would expect substantial genome-wide differentiation due to restricted gene flow. However, a genome-wide F_{ST} scan between P_2–9r and X–9a males revealed no clear signatures of genome-wide differentiation, with overall F_{ST} values remaining low except for the regions of suppressed recombination on chromosomes 3 and 9 (Scarparo et al. 2023). This pattern indicates that while strong epistatic selection, reinforced by mating incompatibilities, maintains LD between supergenes, gene flow remains sufficient to prevent reproductive isolation. Gene flow is facilitated by specific cross-morph matings that avoid lethal recombinants: XX-9a9a queens mated with P_2-9r males and P_2P_2-9r9r queens mated with X-9a males both produce entirely non-mismatched offspring, allowing alleles to move between morphs without fitness costs (Table 2). The system thus appears to represent a stable polymorphism where epistatic incompatibilities generate strong selection against specific recombinant genotypes, while gene flow through compatible matings and counterbalancing fitness benefits preserve both morphs within a single population.

Why did the supergenes not become physically linked?

These findings raise an important evolutionary question: why did the two supergenes not become physically linked, thereby reducing the production of costly lethal mismatches? We propose two non-mutually exclusive explanations.

First, the current system may represent an intermediate evolutionary stage. Physical linkage could evolve if a suitable chromosomal rearrangement (such as a translocation or fusion) arises and becomes established in the population. However, such mutations are rare. The absence of physical linkage may therefore simply reflect insufficient evolutionary time or the failure of appropriate mutations to arise and spread.

Alternatively, physical linkage may not have evolved because the fitness costs of mismatched offspring may be lower than initially expected. Both eggs and early-stage larvae are small and require minimal resources. If lethality occurs during development rather than at the adult stage, as our data suggest, colonies may not suffer substantial resource loss. Indeed, social insects commonly consume eggs and larvae, so developmental mortality may not represent a complete loss of invested resources. Moreover, social insect queens are not limited in egg production throughout their lifespan. Thus, even substantial offspring loss does not necessarily translate to proportional fitness costs. Under this scenario, selection for physical linkage may be weak.

Conclusion

The *F. cinerea* system represents a striking example of how epistatic interactions between supergenes can generate and maintain complex polymorphisms through selection against recombinant genotypes, without requiring physical linkage. Whether this represents a stable evolutionary equilibrium or a transitional stage toward eventual chromosomal fusion remains an open question. The mechanistic basis of these epistatic interactions remains an important avenue for future research. Understanding why specific mismatched combinations show complete lethality (P_2-9a and X-9r in haploid males) while others show reduced viability could reveal the functional relationships between these supergene regions. Overall, this system helps to elucidate both the developmental constraints linking social organization and body size, and the broader evolutionary forces maintaining such costly yet persistent supergene systems.

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Data, Materials, and Software Availability

All data generated in this study are available as supplementary materials. Primer sequences are provided in Table S1. Complete genotyping data for chromosomes 3 and 9 for all analyzed samples will be made available upon manuscript publication.

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Figures and Tables

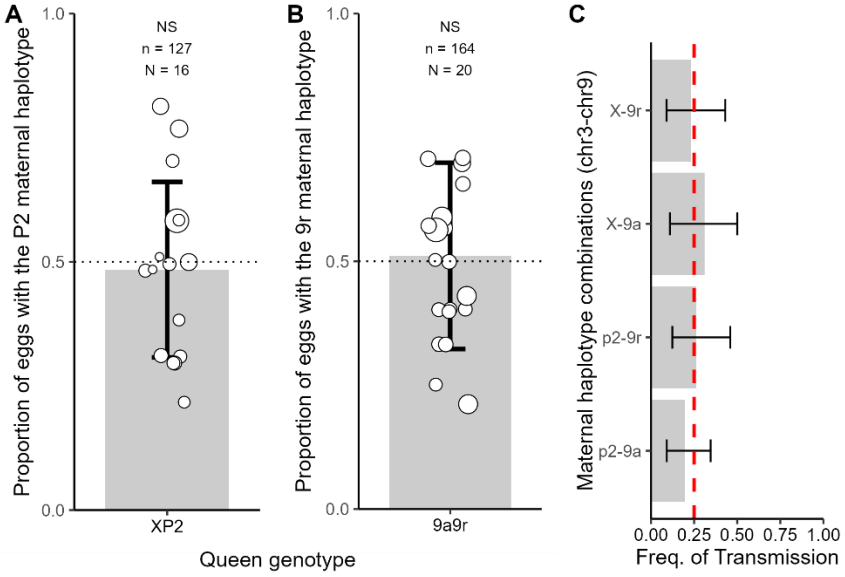

Figure 1. (A-B) Absence of meiotic drive: Maternal P_2 and 9r haplotypes are transmitted to eggs in the expected 1:1 Mendelian ratio, indicating no evidence of meiotic drive. Error bars represent standard deviation, and dot size is proportional to the number of eggs analyzed per queen(C) Independent assortment: Supergene haplotypes on chromosome 3 assort independently from those on chromosome 9, demonstrating that the elevated linkage disequilibrium (LD) between P_2 and 9r is not caused by physical linkage. Barplot shows the multinomial regression model's predicted mean frequency for each haplotype combination (averaged across all queens and mates), with error bars showing the 95% Bayesian credible intervals (CrI).

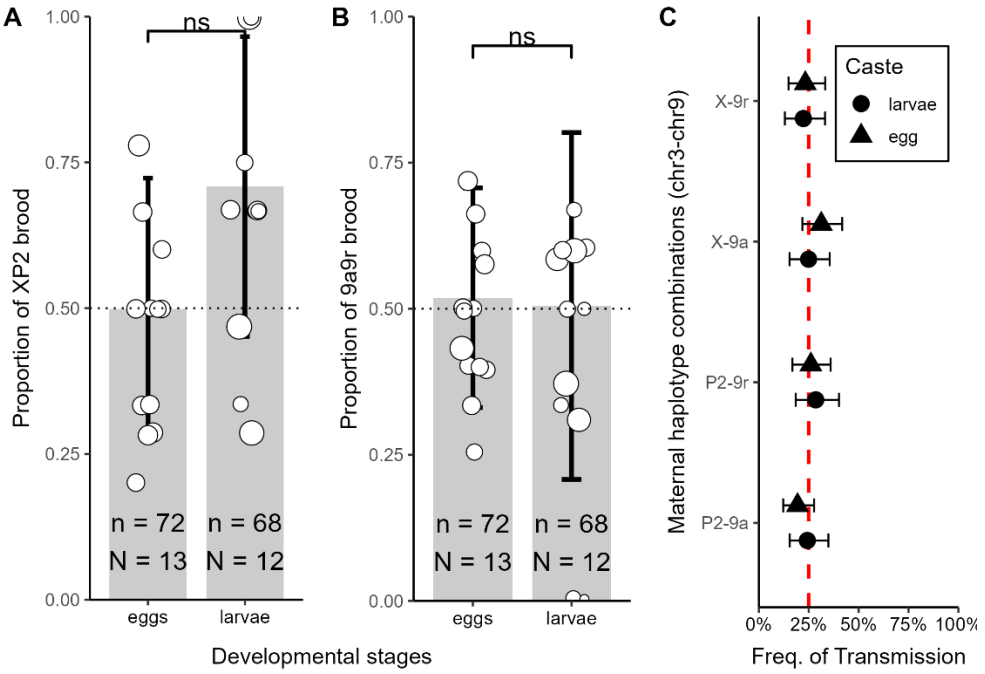

Figure 2. (A-B) Absence of maternal-effect killing: P_2 and 9r do not act as maternal-effect killers, as the proportion of heterozygous brood does not differ between eggs and larvae. Error bars represent standard deviation, and dot size is proportional to the number of eggs analyzed per queen(C) In larvae, the proportions of the four maternal haplotype combinations do not deviate from the expected 1:1:1:1 ratio, and do not differ from transmission observed in eggs, indicating that none of these haplotype combinations cause early mortality. The plot shows the multinomial regression model's predicted mean frequency for each haplotype combination (averaged across all queens and mates), with error bars showing the 95% Bayesian credible intervals (CrI).

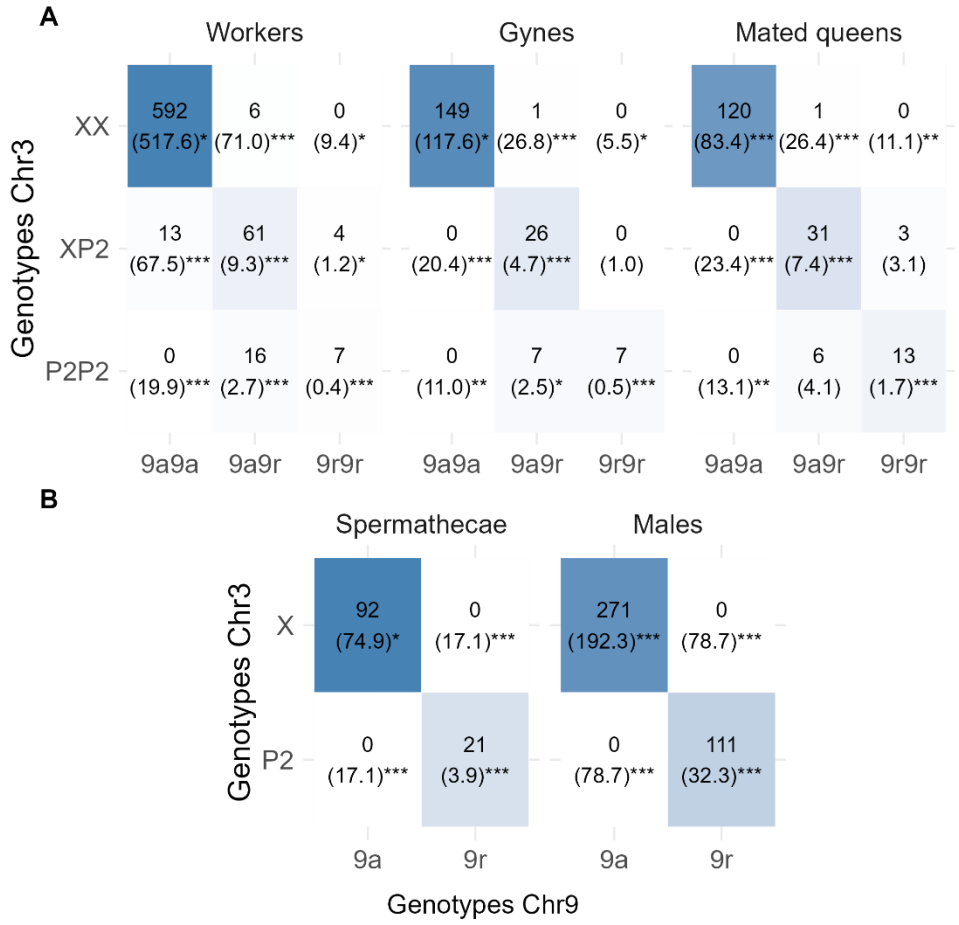

Figure 3. Comparison of expected and observed population-level genotype frequencies in diploid individuals analyzed with RAD-seq by Scarparo et al. (2023)(A): workers, gynes, and mated queens; and haploid individuals (B): spermathecal content and males. We only show the results for single-mated queens for the spermathecal content. Different cell colors reflect the observed frequency of each genotype combination, with more common combinations appearing in dark blue and the least common combinations in white.

Table 1. Overview of *F. cinerea* supergene system. The supergene on chromosome 3 is associated with monogyny (the presence within a colony of a single reproductive queen) and polygyny (the presence of two or more reproductive queens). The supergene on chromosome 9 is associated with sexual body size, where large queens and males are respectively called macrogynes and macraners, and small queens and males are called microgynes and micraners.

Genotype chr3	Genotype chr9	Colony social form	Sexual body size	Notes
M_AM_A M_AM_D M_DM_D	9a9a	Monogyne (=single queen colony)	Macrogynes (=large queens) and macraners (=large males)	
M_AP_1 M_DP_1 P_1P_1	9a9a	Polygyne (=muli queen colony)	macrogynes and macraners	polygyne macrogynes are 5% samller than monogyne macrogynes; polygyne macraners are 2.6% bigger than monogyne macraners
M_AP_2 M_DP_2 P_1P_2 P_2P_2	9a9r 9r9r	Polygyne	Microgynes (=small queens) and micraners (=small males)	microgynes are up to 20% smaller than macrogynes; micraners are ~9% smaller than macraners

Table 2. Offspring viability depends on queen-male genotype combinations. Queens carrying P_2-9r haplotypes produce predominantly viable diploid offspring when mated with P_2-9r males, but largely unviable offspring when mated with X-9a males.

Queen genotypes	Mate genotypes	Offspring genotypes	Expected freq.	Observed fitness outcome
XP_2-9a9r	P_2-9r	XP_2-9a9r	25%	fit
		XP_2-9a9r	25%	fit
		P2P_2-9a9r	25%	fit
		P2P_2-9r9r	25%	fit
P_2P_2-9a9r	P_2-9r	P_2P_2-9a9r	50%	fit
		P_2P_2-9r9r	50%	fit
XP_2-9r9r	P_2-9r	XP_2-9r9r	50%	fit
		P_2P_2-9r9r	50%	fit
XP_2-9a9r	X-9a	XX-9a9a	25%	common in monogyne but rare in polygyne colonies
		XX-9a9r	25%	low survival
		XP_2-9a9r	25%	fit
		XP_2-9a9a	25%	low survival
P_2P_2-9a9r	X-9a	XP_2-9a9a	50%	low survival
		XP_2-9a9r	50%	fit
XP_2-9r9r	X-9a	XX-9a9r	50%	low survival
		P_2-9a9r	50%	fit