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Hybridization and social polymorphism in two ant species

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Abstract

Uncovering the mechanisms maintaining polymorphism within and between species is of high interest in evolutionary biology. Hybridization has been shown to have a great impact on the species evolution. This thesis aims at better understand how polymorphisms are maintained in two ant species, *Formica selysi* and *F. cinerea*. Both are socially polymorphic and harbor a supergene controlling whether colonies are headed by one queen (monogyne) or by multiple queens (polygyne). Previous evidence of hybridization between the two species prompted me to characterize the structure of the hybrid zone. It exhibits a mosaic structure and the distribution of hybrids is asymmetric and skewed towards *F. cinerea*. I investigated potential evolutionary mechanisms limiting gene flow between the two species. Mating occurred assortatively, with a preference for queens and males to mate with conspecifics, but without any genetic incompatibilities detected. Chemical recognition cues were distinct and perceived between species, suggesting species recognition system plays a role in the evolution isolation mechanisms. This first part highlights the implication of the recognition system in the evolution of reproductive isolation in ants. In the third chapter, I explored how distinct dispersal and competition strategies allow both social forms to colonize distinct habitats in sympatry. I found that monogyne colonies are more frequent in less populated and unconnected habitats, whereas polygyne colonies are abundant in young and continuous habitats, suggesting that fine-scale habitat heterogeneity contributes to the coexistence of social forms in sympatry. In the last chapter, I investigated the role of mating system and cyto-nuclear incompatibilities in the maintenance of this social polymorphism. I found evidences suggesting female-mediated gene flow between forms. This second part allows us to learn more about the selective forces at play in the maintenance of social polymorphism. Altogether, this thesis sheds light on evolutionary mechanisms involved in the maintenance of polymorphism both within and between species.

Résumé

Comprendre les mécanismes impliqués dans le maintien du polymorphisme intra- et inter-spécifique est un enjeu de première importance en biologie. Il a été démontré que l'hybridation affecte l'évolution des espèces. Dans cette thèse, je m'intéresse à l'hybridation ainsi qu'au polymorphisme social chez deux espèces de fourmis, *Formica selysi* et *F. cinerea*. Toutes deux possèdent un supergène qui détermine si la colonie contient une reine (monogyne) ou plusieurs reines (polygyne). Des preuves de l'hybridation entre ces deux espèces m'ont encouragé à caractériser génétiquement cette zone hybride. Elle présente une structure mosaïque et la distribution des hybrides est asymétrique, biaisée vers *F. cinerea*. Dans le deuxième chapitre, j'ai étudié les mécanismes d'isolement reproductif entre ces deux espèces. Les reines et les mâles ont ainsi une préférence à s'accoupler avec leurs congénères et aucune incompatibilité génétique n'a été détectée entre les deux espèces. De plus, les indices de reconnaissance chimique sont reconnus par les deux espèces. Cette première partie a ainsi mis en lumière l'implication du système de reconnaissance dans l'évolution de l'isolement reproductif chez les fourmis. Dans le troisième chapitre, j'étudie comment les stratégies de dispersion et de compétition permettent aux deux formes sociales de coloniser des habitats distincts. Les monogynes sont plus fréquentes dans les zones moins peuplées, alors que les polygynes sont abondantes dans les habitats jeunes et continus, suggérant qu'une hétérogénéité d'habitat contribue à la coexistence du polymorphisme. Enfin, dans le dernier chapitre, je me suis intéressé au rôle de la stratégie reproductive et des incompatibilités cyto-nucléaires dans le maintien du polymorphisme social. Il s'avère qu'il y a du flux de gènes lié aux reines entre les formes. Cette deuxième partie a permis d'en apprendre davantage sur les forces sélectives en jeu dans le maintien d'un polymorphisme social. Dans l'ensemble, cette thèse a mis en lumière des mécanismes et des processus qui contribuent au maintien du polymorphisme au sein et entre espèces.

General introduction

Polymorphisms: evolution and maintenance

Polymorphism is the co-existence of two or more genetically determined discrete morphs within a population (Ford 1945), as, for example, males and females of sexually dimorphic species, color morphs in jaguars or in the peppered moth, and blood types. Polymorphism requires a genetic basis and is thus different from discrete phenotypic variation within species caused by environmental factors, or by an interaction between genes and environment. Genetic polymorphism is not necessarily stable, as one of the variants can replace the other, due to directional selection or genetic drift (Lewontin 1974; Nielsen 2005). Alternatively, polymorphism can be maintained by balancing selection across environments and throughout evolution (Ford 1945), resulting in the coexistence of alternative genetic variants at a higher frequency than expected under mutation-drift equilibrium (Fisher 1930). Which mechanisms maintain polymorphism remains an on-going question in evolutionary biology.

In natural populations, balanced polymorphisms have been linked to three types of mechanisms (Charlesworth 2006; Llaurens *et al.* 2017). First, polymorphism can be maintained if heterozygous individuals have a higher fitness than homozygous ones (heterozygous advantage; Fisher 1922). The first empirical evidence for this type of balancing selection was obtained by studying genetic variation at allozyme loci in *Drosophila pseudoobscura* (Lewontin & Hubby 1966). Second, frequency-dependent selection can maintain different alleles in the population, if an allele frequency is negatively associated with its own fitness. An example of this is the obligate disassortative mating associated with self-incompatibility systems in plants, because pollen with a rare allele are more likely to find compatible receptive flower types (Wright 1964). Third, selection varying in time and space can maintain polymorphisms through local adaptation when, the fitness of each haplotype varies according

to ecological conditions (Levene 1953; Bulmer 1972; Kawecki & Ebert 2004), as when selection balances melanic and white forms in the peppered moth *Biston betullaria*. After pollution covered trees in soot during the industrialization, the dark morph increased. Later, the frequency of the dark morph has diminished again, following the decrease of coal use (Berry 1990; Richardson *et al.* 2014).

Hybridization and gene flow

Gene flow underlies fundamental evolutionary processes such as hybridization and speciation. Hybridization occurs when two genetically distinct lineages inter-cross, resulting in the production of interspecific hybrids. The development of modern molecular technics and their widespread use across diverse taxa has shown that gene flow between species is a common phenomenon, with at least 25% of plant species and 10% of animal species displaying hybridization (Mallet 2005; Taylor & Larson 2019).

Hybridization can reduce genetic diversity by mixing and homogenizing genomes that were evolving independently (Barton 2001). Hybrid offspring are often inviable or sterile (Muirhead & Presgraves 2016). If viable, hybrids can fail to exploit the same resources as the parental species or display unusual courtship, reducing their mating success (Arnold 1997; Coyne & Orr 2004). Overall, hybrids tend to have a lower reproductive success (Coyne & Orr 2004; Abbott *et al.* 2013), which will lead to the evolution of stronger mating barriers caused by selection against the hybrids. Preserving genetic integrity by limiting gene flow between distinct lineages is a crucial component of species' evolution. However, there are some cases in which hybridization promotes diversity and leads to adaptive changes.

Adaptive introgression allows species to acquire adaptive variation from a related taxon through hybridization (Hedrick 2013; Abbott *et al.* 2013). This process has been well studied in plants, but has only recently been considered as a source of great evolutionary potential in animals. An

increasing number of studies suggest that hybridization played an important role in the emergence of complex alternative phenotypes, such as HLA in modern humans (Abi-Rached *et al.* 2011), beak traits in Darwin's finches (Grant & Grant 2016) and Müllerian mimicry in *Heliconius* butterflies (Dasmahapatra *et al.* 2012; Pardo-Diaz *et al.* 2012). Introgression could thus have a large impact on the species evolution, either by maintaining polymorphism allowing adaptation or by accelerating the evolutionary change.

Hybrid zones are geographical regions where there is on-going gene flow between two genetically distinct lineages, with the production of viable offspring. The most frequent cause of hybrid zone formation is secondary contact, which occurs when two populations with genetic differences accumulated through geographic isolation cross-fertilize (Barton & Hewitt 1985). On rare occasions, hybrid zones can also result from a primary differentiation. In that case, lineages became differentiated within a population, generally due to an environmental gradient (Bush & Smith 1998). Hybrid zones vary in their geographic structure. First, hybrid zones can be clinal, with parental lineages present on each side and hybrid individuals in the center (Endler 1977; Kawakami & Butlin 2012). Second, hybrid zones can be mosaic, with parental lineages and hybrid individuals distributed in patches without any clinal distribution (Ross & Harrison 2002). The maintenance of a hybrid zone and the associated geographical pattern result from a subtle equilibrium, which depends on the evolution of mating barriers and on selection against hybrids.

Social organization and social polymorphism

Social organization varies widely among species. Eusociality is characterized by the division of reproductive roles, with some individuals monopolizing reproduction. The evolution of this extreme division of reproductive labor is explained by kin selection theory and associated concept of inclusive fitness (Hamilton 1964). In short, non-reproducing individuals increase

their inclusive fitness by contributing to the reproduction of related individuals. Patterns of cooperation and conflict within social groups are thus affected by relatedness among group members, which is shaped by the number of breeders (queens) in a colony and by the number of mating events of the reproductive individuals (Hölldobler & Wilson 1977; Bourke & Franks 1995). The number of reproductive individuals greatly varies both among and within species. In the case of eusocial insects, colonies can host several queens (polygyny) or one queen (monogyny). While monogyny is ancestral, polygyny has evolved independently several times (Boomsma *et al.* 2014; Boulay *et al.* 2014). Species exhibiting variation in social organization, with both single-queen and multiple-queen colonies, are widespread (approx. 15% of Palearctic ant species; Boomsma *et al.* 2014; Boulay *et al.* 2014). These differences in social structure are commonly associated with changes in life-history traits, such as colony size, mating systems, mode of colony reproduction, dispersal abilities and morphology, a phenomenon known as the "polygyny syndrome" (Keller 1993; Bourke & Franks 1995; Boulay *et al.* 2014). Typically, polygyne species are characterized by larger colonies, which produce fewer winged queens, that disperse by foot and start a nest with the help of their sister-workers. Social organization is thus a complex phenotype, involving multiple co-adapted traits which are expressed both at the individual and at the colony level. This raises an interesting question regarding the mechanisms underlying the evolution and maintenance of such polymorphisms, as co-adapted alleles are likely to be disrupted by recombination, creating maladaptive phenotypes (Pinho & Hey 2010).

Supergenes

Supergenes are non-recombining genomic regions with a set of neighboring loci in tight genetic linkage (Fisher 1930; Schwander *et al.* 2014; Thompson & Jiggins 2014; Charlesworth 2016; Llaurens *et al.* 2017). Several mechanisms can suppress recombination between loci in supergenes, including close physical proximity on chromosomes, gene duplications and chromosomal rearrangements such as inversions (reviewed in Schwander *et al.* 2014). The

genes linked in supergenes are inherited as a single locus, which prevents the formation of maladaptive trait combinations, and produces discrete alternative phenotypes within populations (Dobzhansky 1970).

Polymorphic inversions have, since the early development of genetics, been suspected to have an important role in the evolution of genomes (Ford 1966; Kirkpatrick & Barton 2006; Hoffmann & Rieseberg 2008; Kirkpatrick 2010; Thompson & Jiggins 2014). Since the discovery of a chromosomal inversion in *Drosophila* a 100 years ago by Sturtevant (Sturtevant 1921), many supergenes have been found throughout the tree of life (Dobzhansky & Sturtevant, 1938; Thorneycroft, 1975; Joron, 2006; Wang *et al.*, 2013; Purcell *et al.*, 2014). Together, they control complex adaptive phenotypes composed of several behavioral and morphological traits (Schwander *et al.* 2014). In addition to the suppression of recombination linking co-adapted alleles, a non-mutually exclusive hypothesis for the evolution of supergenes is that they spread selfishly (Avril *et al.* 2020). Indeed, due to the lack of recombination, co-transmitted alleles are likely to contain alleles interacting to favor their own transmission over that of alternative alleles (i.e. transmission ratio distorters; Burt & Trivers 2006). Supergenes can favor their own transmission, for example, by meiotic drive (i.e. the overrepresentation of one allele in the gametes), by green beard effects (i.e. one individual carrying an allele can recognize and favor individuals that also carry this allele) or by maternal killing (i.e. parental genetic element can cause the death of the progeny that do not inherit it; Burt & Trivers 2006).

Since the advent of the genomic era, numerous studies have shown the wide implication of supergenes in the co-existence of complex phenotypes. Specifically, supergenes have been linked to underlie multiple polymorphisms, including Müllerian mimicry in *Heliconius* butterflies (Joron *et al.* 2006), shell patterns in the land snail *Cepea nemoralis* (Murray & Clarke 1976; Richards *et al.* 2013), autosomal drivers in the house mouse (Lyon 2003), alternative mating behavior in birds (Küpper *et al.* 2016; Tuttle *et al.* 2016), ecological forms and

heterostyly in plants (Li *et al.* 2016) and sperm morphologies in the zebra finch *Taeniopygia guttata* (Kim *et al.* 2017; Knief *et al.* 2017). In social Hymenoptera, supergenes have been associated with elevation in honeybees (Wallberg *et al.* 2017) and with the control of social organization (colony queen number) in ants (Wang *et al.* 2013; Purcell *et al.* 2014b; Braims 2015; Brelsford *et al.* 2020; Yan *et al.* 2020).

The convergent supergenes underlying social organization in the two most studied socially polymorphic ant species, *Solenopsis invicta* and *Formica selysi*, are nearly chromosome-wide and contain hundreds of genes (~13Mbp and ~600 genes; Wang *et al.* 2013; Brelsford *et al.* 2020). Those two "social supergenes" are ancient and evolved independently in each lineages (Brelsford *et al.* 2020; Yan *et al.* 2020). In the *Formica* group, the non-recombining region is conserved across 14 socially polymorphic species and evolved 20 to 40 million years ago, whereas in the *Solenopsis* clade, it is conserved across six species that diverged half a million years ago (Brelsford *et al.* 2020; Yan *et al.* 2020). These supergenes also harbor selfish genetic elements. In *S. invicta*, the polygyne recessive haplotype Sb causes a green beard effect (Keller & Ross 1998; Ross & Keller 1998). In *F. selysi*, the polygyne Sp haplotype is a maternal killer (Avril *et al.* 2020). The fact that these two supergenes are not homologous but display many similarities provides a great opportunity to compare the two clades and to understand the evolutionary routes leading to the evolution and maintenance of supergenes.

Consequences of hybridization in ants

In eusocial insects, hybridization can impact the fitness and phenotype of individuals and colonies, while the consequences of hybridization will depend on the species life-history and genetic traits. Ants present several characteristics that make them valuable models to study the hybridization process and its consequences. An increasing number of cases indicate that, in ants, pre-mating isolation mechanisms are scarce and hybridization is more common than

previously thought (Feldhaar *et al.* 2008). In some species, up to half the young queens mate at least once with a heterospecific (Seifert 1999). However, ant queens are expected to be highly choosy when picking a mate, which would result in low hybridization rate, as they mate in a single event at the beginning of their lives and store sperm to fertilize eggs for several years. High hybridization rates may be explained by several phenomena, including the paucity of conspecific mate (Nonacs 2006) and the reduced diversity in morphology and behavior in males (Feldhaar *et al.* 2008; Boudinot 2012; Divieso *et al.* 2020). In addition, hybridization can result from a recent increase in range of many species, either human-mediated (Seifert 1999) or because of post-glacial recolonization (Pusch *et al.* 2006). Despite its commonness, the consequences of hybridization in ants and other social Hymenoptera have received surprisingly little attention.

Ants are haplodiploids, with males developing from unfertilized eggs, whereas diploid females are produced via sexual reproduction, with equal genetic contribution of both parents. In the context of hybridization, the first generation resulting from an interspecific mating consists of pure-bred males but hybrid females (workers and queens). However, in the second generation of hybrids, introgressed alleles will be exposed to selection in haploid males, leading to high mortality and to a fast purging of recessive deleterious mutations. On their side, females, as diploid, will be less exposed to the deleterious consequences early in the hybridization process. The distinct genetic consequences of recessive mutations for males and females can lead to hybrid zones composed of hybrid females and non-hybrid males (as seen in Kulmuni & Pamilo 2014). In some extreme cases, heterospecific mating may result in peculiar reproductive system such as hybridogenesis, whereby workers are systematically produced by hybridization between two lineages, whereas males and queen are pure-bred (Lavanchy & Schwander 2019). Yet, fertile hybrid queens and males are not common in nature, suggesting a high cost of hybridization in social Hymenoptera (Feldhaar *et al.* 2008; Kulmuni *et al.* 2010).

As workers generally do not reproduce, some classical consequences of hybridization such as hybrid infertility will have little effect on them. In hybridization-linked caste systems, all workers are obligatory hybrids, while males and queens are pure-bred, as in the *Solenopsis geminata* x *Solenopsis xyloni* case (Helms Cahan & Vinson 2003). In addition, hybrid vigor (also known as heterosis, i.e. the increase function of biological traits in hybrid offspring) is often accompanied by deleterious hybrid traits such as sterility. However, in ants, hybrid vigor can confer colony-level benefits when affecting workers. As an example, hybrid workers between *Solenopsis invicta* and *S. richteri* were more tolerant of low temperatures than workers of either pure species, potentially providing a selective benefit in their introduced range in Mississippi, USA compared to their native range in South America (James *et al.* 2002).

Hybridization in ants can thus have an impact at the colony level. By increasing the genetic diversity in the colony, it may mitigate the effects of parasitism (Hughes & Boomsma 2004; Cremer *et al.* 2007). Indeed, a colony with low genetic diversity may be less resistant to a pathogen or parasite (Schmid-Hempel 1995, 1997). Multiple life-history and social traits will influence whether hybridization impacts the survival of a colony. For example, polyandry (i.e. queen having multiple mates) may dilute some negative consequences of hybridization, as a high number of mates reduces the probability of mating with a heterospecific and reduces the proportion of hybrid offspring (workers or queens) in the colony. Likewise, polygyny (i.e multiple queens in a colony) may reduce the colony-level costs of heterospecific matings if those represent a minority of cases among reproductive queens. In addition, some queens in polygyne colonies may mostly contribute to the production of workers (Helms Cahan & Vinson 2003), minimizing some consequences of hybridization at the colony level.

Study species

The Alpine silver ant, *Formica selysi* (Bondroit 1918), is a pioneer ant that lives in heterogenous floodplains across Europe, from the Alps to the Pyrenees (Seifert 2002). The ecology, social structure and behavior of *F. selysi* have been studied in detail for 20 years (e.g Chapuisat *et al.* 2004; Meunier & Chapuisat 2009; Meunier *et al.* 2010). Its habitat usually encompasses open, disturbed and sandy areas along rivers. *F. selysi* preys on various arthropods and collects honeydew from aphids (Keller & Zettel 2001). Colonies can survive occasional floods by building living rafts and floating to the bank (Lude *et al.* 1999; Purcell *et al.* 2014). *Formica selysi* is a socially polymorphic species, with populations containing monogyne (i.e single-queen) and polygyne (i.e. multiple-queen) colonies in sympatry (Chapuisat *et al.* 2004). The social structure of the colonies appears stable through time at the population level (Purcell & Chapuisat 2013). However, the proportion of monogyne colonies within populations increases with altitude, which likely result from different ecological optima between social forms (Purcell *et al.* 2015). In line with this hypothesis, queens of monogyne colonies survive better cold and long winters (De Gasperin *et al.* 2020). Additionally, the monogyne and polygyne forms may play distinct roles in a source-sink dynamic (Pulliam 1988; Hanski 1998). If monogyne queens are better dispersers, they may be favored in colonizing harsh and unstable high elevation habitats.

Social organization in *F. selysi* is controlled by a supergene with two haplotypes, Sm and Sp (Purcell et al., 2014). All individuals in a monogyne colony bear two copies of the Sm haplotype, whereas in polygyne colonies, all individuals carry at least one copy of the Sp haplotype. In that case, workers and queens are Sp/Sp or Sp/Sm, and males Sp. In addition, and contrary to other organisms bearing a supergene, the mutant homozygous (Sp/Sp) is viable in *F. selysi*, whereas Sm male and Sm/Sm female offspring of heterozygous queens in polygyne colonies do not develop due to a maternal effect killing (Avril *et al.* 2020). Genetic analyses

revealed that polygyne queens mate with more related males than monogyne queens, and that heterozygous Sm/Sp queens are more likely to mate multiple times (Avril *et al.* 2019a). Yet, genetic differentiation between social forms at neutral markers outside the supergene is weak, suggesting ongoing gene flow between social forms (Chapuisat *et al.* 2004; Avril *et al.* 2019a). This gene flow is highly asymmetric, occurring mostly from the monogyne to the polygyne social form. It can be explained by an asymmetric assortative mating pattern (Avril *et al.* 2019a). Indeed, queens in polygyne colonies in the wild predominantly mate with Sp males (in 77.1% of cases; Avril *et al.* 2019). In contrast, queens in monogyne colonies mate exclusively with Sm males (Purcell *et al.* 2014; Avril *et al.* 2019a). In line with this result, no genetic or behavioral incompatibilities between the two forms have been detected (Reber *et al.* 2010; Avril *et al.* 2019b - Appendix I). Across numerous field surveys, no monogyne colonies headed by a Sm/Sm queen mated to a Sp male could has been observed (Purcell *et al.* 2014; Avril *et al.* 2019a). The absence of this cross in mature field colonies is intriguing, as Sm/Sm females and Sp males mate successfully under laboratory conditions and produce viable offspring (Reber *et al.* 2010; Avril *et al.* 2019b - Appendix I).

Alternative social organization in *F. selysi* is associated with many differences in life-history traits (Rosset & Chapuisat 2007). Nest density, colony size, egg, queen and worker sizes, fecundity, survival rate in absence or presence of a pathogen, development time, resistance to harsh condition and chemical profiles are significantly different between the monogyne and the polygyne social forms (Schwander *et al.* 2005; Reber *et al.* 2008, 2010; Meunier *et al.* 2010, 2011; Purcell & Chapuisat 2012; Purcell *et al.* 2015; Avril *et al.* 2019a; De Gasperin *et al.* 2020). Despite all the attention paid to this species, several aspects of its ecology and dispersal remain to be investigated.

The second study species, *Formica cinerea* (Mayr 1853), occurs in sandy habitats, from costal to pine forests and town. It is broadly distributed across Europe, from Northern Spain to Siberia

and from Greece to Finland (Seifert 2002). Monogyne and polygyne colonies frequently co-occur, but in some cases, populations contain only one or the other form, particularly in Northern Europe (Goropashnaya *et al.* 2001; Zhu *et al.* 2003). This social polymorphism is linked to a supergene that is homologous to the one of *F. selysi* (Brelsford *et al.* 2020). Populations frequently develop into large and populous polydomous systems of interconnected nests (Seifert 2002). It is a predatory species that scavenges, but also feeds on honeydew. In addition, *F. cinerea* is a host for slave maker ants (Chernenko *et al.* 2013) and butterflies (Obregón *et al.* 2015). Its ecology, social structure and behavior have been well studied in the North of Europe (e.g Lindström *et al.* 1996; Czechowski 2001; Goropashnaya *et al.* 2001; Zhu *et al.* 2003; Sirvio & Pamilo 2010; Markó & Czechowski 2012), yet we lack data on its genetic and population structure, ecology and social structure in Central Europe.

Aim of the PhD

The aim of my PhD is to better understand how polymorphisms are maintained in two species, *F. selysi* and *F. cinerea*, a pair of socially polymorphic ants that naturally hybridize. The first part of this thesis characterizes the genomic structure of the hybrid zone and identifies reproductive barriers, using genomic and genetic data, behavioral experiments and chemical analyses. In the second part of this thesis, I combine fieldwork and genomic approach to investigate the effects of habitat, dispersal and gene flow on the distribution and maintenance of a supergene-controlled social polymorphism.

In the first chapter I characterized a hybrid zone between *F. selysi* and *F. cinerea* in Switzerland. A previous biogeographical study based on microsatellite and mtDNA had revealed that *F. selysi* and *F. cinerea* hybridize (Purcell *et al.* 2015), which prompted us to investigate the structure of a hybrid zone. Using a genotyping-by-sequencing (GBS) approach, we characterized the zone, assessed the frequency of hybrid workers, inferred the presence of

hybrid males and females and investigated whether hybridization is influenced by colony social organization (Brelsford *et al.* 2020). In addition, we compared the cuticular hydrocarbon (CHC) profiles and aggression levels between the two species. Overall, the hybrid zone exhibits a mosaic structure with populations formed by *F. selysi*, by *F. cinerea*, by hybrids or by a mix. The distribution of hybrids was asymmetric and skewed towards *F. cinerea*. In addition, both species display strongly differentiated cuticular hydrocarbon profiles and a high level of interspecific aggression, revealing efficient species recognition.

In the second chapter of my PhD work, I investigated potential evolutionary and proximate mechanisms maintaining species boundaries between *F. cinerea* and *F. selysi*. Taking advantage of the hybrid zone described in Chapter 1, I conducted field surveys to test for temporal isolation, and mating assays to investigate whether assortative mating limits gene flow between the two species. I recorded offspring production from queens mated with a conspecific or heterospecific male to detect genetic incompatibilities between species. Finally, to gain insights into species recognition mechanisms, I tested workers behavior in dyadic encounters and then analyzed their CHC profile. I showed that mating occurs assortatively, with a strong preference for queens and males to mate with conspecifics. In addition, I did not detect post-mating barriers caused by genetic incompatibilities. CHC profiles strongly differed between species and workers showed good recognition abilities. This allowed me to discuss the implication of the advanced recognition system of ants in the evolution of pre-mating isolation mechanisms.

In the third chapter, I investigated how the social forms of *F. selysi* are distributed at a fine spatial scale. It has been generally thought that each social form exhibits distinct dispersion and colonization abilities. To investigate if that relates to habitat heterogeneity, I sampled plots with varying ecological and connectivity characteristics. I compared the frequency of monogyne and polygyne colonies across landscapes and showed that the two forms occupy distinct habitat type

associated with distinct connectivity. Single-queen colonies mostly occupied unconnected and less populated habitat, whereas multiple-queen colonies were abundant in populated, young and continuous habitat. This allowed me to discuss how fine-scale spatial heterogeneity may favor the coexistence of alternative social forms through spatially varying selection on colonization and competition traits.

Finally, in the fourth chapter of this thesis, I investigated the role of mating system and potential cyto-nuclear incompatibilities in the maintenance of a supergene-mediated social polymorphism. Taking advantage of complete genomes of *F. selysi*, I investigated whether mitochondrial lineages are specific to social forms, and if they are co-inherited with the supergene, or occasionally segregate independently. I found that the mitogenomes of *F. selysi* present low genetic variation compared to other Hymenopter. In addition, there is no or little mitochondrial genetic differentiation between social forms, indicating at least occasional female-mediated gene flow and that the maternal killing effect is likely not due to cyto-nuclear incompatibilities. This chapter provides new insights into the mechanisms mediating gene flow between the two social forms involved in the maintenance of a social polymorphism.

Altogether, the first part of this thesis improves our understanding of the hybridization process in ants and suggests that their well-developed recognition system may facilitate the evolution of pre-mating barriers, even in the absence of strong hybridization costs. The second part explores the role of dispersal and gene flow in the maintenance of a social polymorphism. Overall, this work sheds the light on the evolutionary mechanisms and forces involved in the maintenance of polymorphisms both within and between species.

Chapter 1

Ants exhibit asymmetric hybridization in a mosaic hybrid zone

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Authors' contributions

JP, MC and AB planned this study. JP and AB collected samples for genotyping-by-sequencing and prepared the GBS library. AB analysed the next generation sequencing data with help from SZ. SZ collected and genotyped workers from additional colonies in Branson and Riddes. AA and RT carried out the CHC analysis and the aggression tests and analysed the data, under the supervision of JP and MC. JP, MC and AB wrote the manuscript with editorial input from all authors.

Abstract

Research on hybridization between species provides unparalleled insights into the pre- and post-zygotic isolating mechanisms that drive speciation. In social organisms, colony-level incompatibilities may provide additional reproductive barriers not present in solitary species, and hybrid zones offer an opportunity to identify these barriers. Here, we use genotyping-by-sequencing to sequence hundreds of markers in a hybrid zone between two socially polymorphic ant species, *Formica selysi* and *Formica cinerea*. We characterize the zone, determine the frequency of hybrid workers, infer whether hybrid queens or males are produced, and investigate whether hybridization is influenced by colony social organization. We also compare cuticular hydrocarbon profiles and aggression levels between the two species. The hybrid zone exhibits a mosaic structure. The asymmetric distribution of hybrids skewed toward *F. cinerea* suggests a pattern of unidirectional nuclear gene flow from *F. selysi* into *F. cinerea*. The occurrence of backcrossed individuals indicates that hybrid queens and/or males are fertile, and the presence of the *F. cinerea* mitochondrial haplotype in 97% of hybrids shows that successful F1 hybrids will generally have *F. cinerea* mothers and *F. selysi* fathers. We found no evidence that social organization contributes to speciation, since hybrids occur in both single-queen and multiple-queen colonies. Strongly differentiated cuticular hydrocarbon profiles and heightened interspecific aggression further reveal that species recognition cues are both present and perceived. The discovery of fertile hybrids and asymmetrical gene flow is unusual in ants, and this hybrid zone will therefore provide an ideal system with which to investigate speciation in social insects.

Introduction

Eusocial insects form several speciose clades, yet researchers still know little about the reproductive barriers leading to speciation in these lineages. Due to their social group structure, speciation and reinforcement processes in eusocial organisms have the potential to differ from those of non-social organisms (e.g. Kulmuni *et al.* 2010; Kulmuni & Pamilo 2014). Individual-level incompatibilities, including pre- and post-zygotic isolating mechanisms, should contribute to reproductive barriers in both social and solitary organisms. Social species could also have novel barriers due to colony-level incompatibilities. These may include diverging social strategies, such as transitions toward increasing queen numbers, which are often accompanied by a shift from nuptial flight mating to intranidal mating (e.g. Seifert 2010). Colony-level incompatibilities may also involve intra-colony conflicts between nestmate queens mated with conspecific *versus* heterospecific males (Helms Cahan & Vinson 2003). More research is needed to determine the prevalence of colony-level mechanisms in speciation and reproductive isolation (Steiner *et al.* 2011).

Hybrid zones can provide insights into reproductive barriers that develop between diverging species (reviewed by Abbott *et al.* 2013). In eusocial insects, hybridization is relatively common and widespread (reviewed by Feldhaar *et al.* 2008), with well-documented examples between subspecies of honeybees (Rinderer *et al.* 1991; Chávez-Galarza *et al.* 2015), in termites (Lefebvre *et al.* 2008), and in many ant genera (e.g. Ross *et al.* 1987; Shoemaker *et al.* 1994; Julian *et al.* 2002; Helms Cahan & Keller 2003; Kulmuni *et al.* 2010; Steiner *et al.* 2011; Kulmuni & Pamilo 2014). Because of the haplodiploid genetic system, hybridization can lead to unusual genetic patterns in Hymenoptera. As haploids, males are particularly likely to show hybrid incompatibilities (Schilthuizen *et al.* 2011), and some species have evolved elaborate mechanisms to avoid producing hybrid males (e.g. Kulmuni *et al.* 2010; Kulmuni & Pamilo 2014). In other social Hymenoptera, hybridization has been coopted to generate a genetic caste

determination mechanism, with hybrid offspring becoming sterile workers and intra-lineage matings producing sexual females (Helms Cahan & Keller 2003; Helms Cahan & Vinson 2003; Schwander *et al.* 2007; Leniaud *et al.* 2012).

When fertile hybrid sexuals are produced, what factors preserve species differences? Some hybrid zones in *Solenopsis* ants exhibit ongoing gene flow between species (e.g. Shoemaker *et al.* 1996). In these systems, hybrids are relatively rare and F1 hybrids are more common than backcrosses, suggesting that strong selection against hybrid queens and males helps to maintain the hybrid zones. Systems like these, wherein some gene flow is maintained between divergent lineages, provide an ideal opportunity to study the process of speciation in eusocial organisms, and to examine whether it differs from non-social organisms.

Here, we investigate a hybrid zone between two species of ants, *Formica selysi* Bondroit and *Formica cinerea* Mayr. *Formica cinerea* is broadly distributed across Europe, extending into western Siberia. *Formica selysi* is restricted to the Alps and Pyrenees, where it generally lives near streams and rivers (Seifert 2002). Variation in social organization has been extensively studied in *F. selysi*. Colonies headed by a single queen (=monogynous) and colonies containing multiple queens (=polygynous) are found in the same populations (Chapuisat *et al.* 2004; Purcell & Chapuisat 2013). Moreover, colony organization in *F. selysi* is associated with a supergene, i.e. a large group of linked genes showing two well-differentiated haplotypes, Sm and Sp (Purcell *et al.* 2014b). Individuals from monogynous colonies have a Sm/Sm or Sm genotype (females and males, respectively) at this supergene, whereas females from polygynous colonies have a Sp/Sp or Sp/Sm genotype and males have a Sp genotype (Purcell *et al.* 2014b, 2015). Social organization is also polymorphic in *F. cinerea*, with some populations in northern Europe containing only monogynous and weakly polygynous colonies, and others containing highly polygynous colonies (Goropashnaya *et al.* 2001).

To characterize the genetic structure of the contact zone between these two species along the Rhône river in Vaud and Valais, Switzerland, we surveyed hundreds of nuclear markers throughout the genome (obtained through genotyping-by-sequencing, GBS; Parchman *et al.* 2012; Purcell *et al.* 2014c; Brelsford *et al.* 2016). In addition, we genotyped a diagnostic single nucleotide polymorphism (SNP) in the mitochondrial COI gene. We had four primary goals: 1) Investigate the frequency of hybrids between *F. selysi* and *F. cinerea*; 2) Determine whether hybrid queens or males ever reproduce in this system; 3) Examine whether any portion of the genome introgresses across this zone; and 4) Ask whether gene flow is limited to one social form (i.e. monogynous or polygynous colonies). Secondarily, we investigated the strength of species discrimination in the contact and in allopatric zones, by comparing cuticular hydrocarbon profiles and measuring intra- and interspecific worker aggression. Taken together, these analyses provide insights into the structure of this hybrid zone and identify some factors that may prevent these two closely related species from collapsing into a single species.

Methods

A recent population genetic analysis provided preliminary evidence of the presence of a contact zone between two divergent genetic lineages in the Rhône valley (Purcell *et al.* 2015). An independent morphology assessment carried out by Dr. Bernhard Seifert (personal communication), revealed that these genetic lineages are in fact two closely related species, *Formica selysi* and *Formica cinerea.* Within the Alps, our surveys revealed that these species differ in their distribution: *F. selysi* is found predominantly north and west of the main range of the Alps (the two mitochondrial lineages from the Mediterranean and North Sea drainage basins, as reported by Purcell *et al.* (2015), were confirmed to be *F. selysi*), while *F. cinerea* is found primarily south and east of the central Alps (in the Adriatic Sea drainage basin, Purcell *et al.* 2015; this study). *Formica selysi* and *F. cinerea* co-occur in several populations along the

Rhône river in Valais and Vaud, Switzerland, which prompted us to investigate whether they hybridize in this area.

Genetic characterization of the hybrid zone

We used a GBS approach to sequence loci distributed across the genome, following the protocol from Brelsford *et al.* (2016). The combination of enzymes selected (SbfI and MseI) produces a lower marker density, but allows more individuals to be multiplexed, than our previous work in this system (Purcell *et al.* 2014b). We genotyped 208 individual workers, each from a different colony, from 21 populations in the putative hybrid zone (Fig. 1a). We processed the GBS data using Bowtie 2.2.0 (Langmead & Salzberg 2012) to align the sequences to the reference genome of *F. selysi* (Purcell *et al.* 2014b) and SAMtools/BCFtools (Li *et al.* 2009) to identify SNPs and call genotypes. We then used VCFtools 0.1.11 (Danecek *et al.* 2011) to filter the resulting genotypes based on quality score (minimum 20), missing data (maximum 0.20 per locus), minor allele frequency (minimum 0.05), and to exclude indels. This resulted in 493 polymorphic loci used in subsequent analyses. 21 of these loci were located within the supergene associated with social organization (Purcell *et al.* 2014b).

To assess the admixture of individuals and genetic structure of populations, we applied a Bayesian clustering approach to the full GBS data (493 loci), implemented in STRUCTURE 2.3.2 (Pritchard *et al.* 2000). The model was replicated 10 times for each K-value from 1 to 10, with a burn-in period of 50,000 iterations, followed by 100,000 iterations per test. We used the Admixture model setting. We inferred the number of genetic clusters in our data following Evanno *et al.* (2005). STRUCTURE admixture coefficients (K = 2) were used to infer ancestry for each individual (hereafter "hybrid index" or "HI"). To assess the haplotypes at the supergene for social organization, we reran the STRUCTURE analyses using the 21 SNPs located within this supergene.

We then identified species-diagnostic nuclear markers (n = 33, on 30 unique scaffolds; defined as frequency differences > 0.95 between allopatric *F. selysi* and *F. cinerea*). We carried out a genomic cline analysis using one diagnostic marker per scaffold, as implemented in the HIest package in R 3.0.2 (Fitzpatrick 2013; R Core Team 2014). Genomic cline analysis examines the relationship between locus-specific and genome-wide ancestry, seeking to identify genetic markers that are more likely or less likely than the genome-wide average to introgress between hybridizing taxa (Szymura & Barton 1991; Gompert & Buerkle 2009). HIest fits genotypic data to genome-wide hybrid index with a logit-logistic model, using maximum likelihood. The hybrid index values used in this analysis were based on STRUCTURE ancestry estimates from the full dataset of 493 loci.

To further characterize the hybrid zone and increase our sample size for cuticular hydrocarbon and behavioral analyses, we genotyped some of the individuals from the GBS sample, as well as individuals from additional colonies, at a mitochondrial SNP. This SNP, located in the COI gene, was species diagnostic for *F. selysi* and *F. cinerea* and is informative for the maternal ancestry of hybrids (Purcell *et al.* 2015). We first genotyped 70 individuals belonging to the GBS sample and coming from thirteen populations located within the contact zone (Table S1). We then sampled workers from 82 additional colonies in two mixed populations. These populations are Branson (46° 7’ 27.16” N, 7° 5’ 8.38” E) and Riddes (46° 10’ 42.89” N, 7° 13’ 7.90” E). We genotyped four workers per colony at the mitochondrial SNP, which allowed us to identify a sample of colonies for subsequent assessment of cuticular hydrocarbon differentiation and aggression (see below). We also assessed the social organization of these colonies by genotyping two workers per colony at two distinct genetic markers, each diagnostic for the supergene haplotype in both species (Sm vs Sp; Purcell *et al.* 2014c; diagnostic in *F. cinerea:* Purcell & Brelsford, unpublished data; Box S1).

Species discrimination: cuticular hydrocarbons and aggression

In order to evaluate the strength of species discrimination in sympatry and allopatry, we collected workers from two populations where both species occur (Branson and Riddes) and two populations away from the contact zone: Aigle for *F. cinerea* (46° 19' 8.72" N, 6° 55' 59.59" E, but note that some individuals in this population were admixed with up to 15% F. selysi ancestry) and Finges for *F. selysi* (46° 18' 48.10" N, 7° 37' 11.35" E). We selected only polygynous colonies, in order to exclude any confounding effect due to phenotypic differences between the monogynous and polygynous social forms (Schwander *et al.* 2005; Rosset & Chapuisat 2007; Rosset *et al.* 2007). We measured two phenotypic traits linked to species discrimination: cuticular hydrocarbon profile and aggression between non-nestmate workers.

Cuticular hydrocarbons

We assessed the cuticular hydrocarbon profile of groups of 10 workers per colony from four *F. cinerea* colonies (and/or *F. cinerea*-like hybrids) and six *F. selysi* colonies. The worker groups were frozen at -20° C the day before the GC-MS analysis. We extracted cuticular components by immersing the 10 workers in 150 µl of pentane solution (93.75 parts pentane, 6.25 parts internal standard) for 15 minutes, and transferring 100 µl of this pentane solution to a new vial. We injected 4 µL of the solution into a Thermo Scientific ™ gas chromatograph with an internal standard consisting of alkane standard solution C8-C20 (40 mg/L) and *n*-Pentane (Sigma). We operated the gas chromatograph in splitless injection mode, and used helium as the carrier gas. We set the oven temperature to 70°C and ramped the temperature at 10°C/minute to a final temperature of 320°C, which was held for 15 minutes. The position and corrected height of each CHC peak was assessed in Xcalibur 1.4 SR1 ™ (Thermo Scientific).

Aggression between workers

Ant aggressiveness was measured by monitoring five-minute pairwise interactions between non-nestmate workers. We tested the following combinations of workers: same species, same population; same species, different populations; different species, same population; different species, different populations. At least 24 hours before the test, workers were marked with a dot of paint on the thorax, with the colour randomized with respect to species and population. Pairs of ants were transferred to a Petri dish side-lined with Fluon. We filmed the interactions for five minutes using a webcam (Logitech C910). For each interaction, aggressiveness was defined on the following scale: 0 = no contact, 1 = antennation, 2 = mandible open when workers are adjacent, 3 = biting, 4 = biting plus acid ejection posture. For each aggression test (n = 94), we retained the highest aggression score (Rosset *et al.* 2007). In interspecific tests, we attempted to identify the individual initiating aggressive interactions of score 3 or 4 (n = 29 interactions). Scoring was carried out by two observers who were blind with respect to the origins of each combination, with a subset of cases scored by both to ensure consistency.

Statistical analysis

We compared the cuticular hydrocarbon profiles of *F. selysi* and *F. cinerea* colonies using a principal component analysis (PCA), implemented in R 3.1.1. To assess the statistical significance of differences in aggression within and between species, and within and between populations, we performed permutation tests using the *boot* and *coin* packages in R. We assessed whether one species was more likely to initiate aggression than the other using a binomial test.

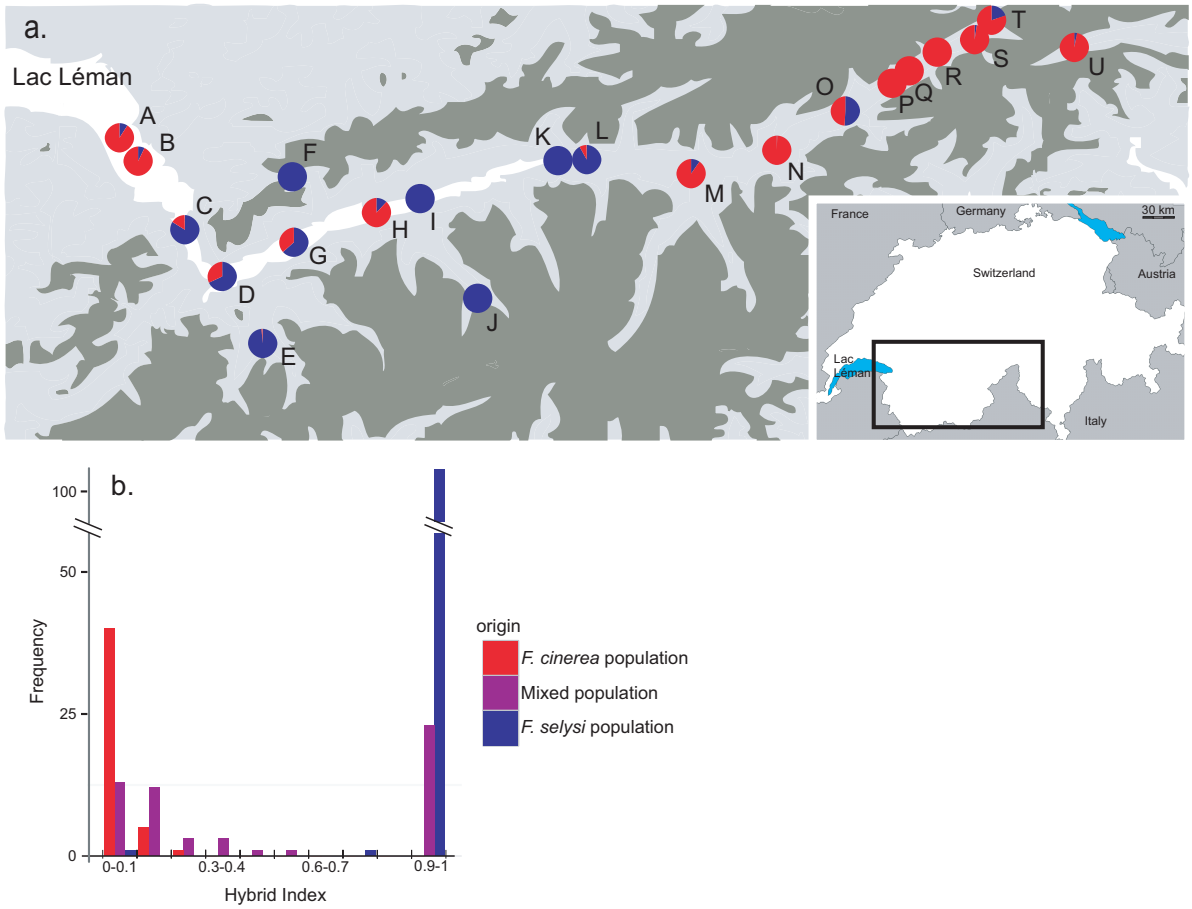

Figure 1. (a) Map of the genetic background of each population. *F. cinerea, F. selysi,* and their hybrids display a mosaic distribution across the Swiss Rhône valley. The pie charts represent the proportion of *F. selysi* (blue) and *F. cinerea* (red) genetic background, estimated from the GBS-based STRUCTURE analysis of 493 SNP markers. Locality names are as follows: A: Noville, B: Aigle, C: St. Maurice, D: Martigny, E: Orsieres, F: Derborence, G: Riddes, H: Conthey, I: Sion, J: Les Hauderes, K: Finges, L: Leuk, M: Visp, N: Naters, O: Fiesch, P: Blitzingen, Q: Biel, R: Goms, S: Ulrichen, T: Obergestein, U: Nufenen. (b) Distribution of hybrid indexes. The distribution is strongly bimodal, with the majority of individuals sampled being predominantly *F. selysi* or *F. cinerea.* These individuals were sampled from populations containing a mostly *F. selysi* genetic background (blue bars, less than 10% admixture with *F. cinerea* at the population level), a mostly *F. cinerea* genetic background (red bars, less than 10% admixture with *F. selysi* at the population level), or a mix of the two backgrounds (purple bars). Both intermediate hybrids and backcrosses are observed in this sample; the distribution of backcrosses is asymmetric and biased towards the *F. cinerea* genetic background.

Results

Genetic characterization of the hybrid zone

Our genomic analyses confirmed the presence of a hybrid zone between *Formica selysi* and *Formica cinerea* in the Swiss Rhône valley, and revealed that the zone has a mosaic structure. Ten sites contained predominantly *F. cinerea* colonies, six sites contained *F. selysi* colonies, and five sites contained colonies of each species as well as hybrids (Fig. 1a). STRUCTURE admixture coefficients of workers in mixed populations, based on 493 polymorphic nuclear loci in one worker per colony, were bimodally distributed, indicating that this sample was composed of mainly pure *F. cinerea* (n = 43) and *F. selysi* (n = 124), with 20% of individuals (n = 41) having a hybrid index (HI) between 0.05 and 0.95 (Fig. 1b). Few of these hybrids had HI values close to 0.5, as would be expected for first-generation hybrids. The presence of backcrosses in this distribution clearly demonstrates that hybrid queens and/or males can produce viable offspring in this system.

Overall, we identified a signature of asymmetrical hybridization, with most sampled hybrids exhibiting a HI between 0.05 and 0.5 (*F. cinerea*-like hybrids; Fig. 1b). The paucity of individuals with HI between 0.5 and 0.95 (*F. selysi*-like hybrids) suggests a pattern of genome-wide introgression from *F. selysi* into *F. cinerea.* The genomic cline analysis of 30 nuclear loci identified two markers as exceptions to this genome-wide pattern (Fig. 2). In these two instances, *F. selysi* alleles were underrepresented in *F. cinerea*-like hybrids relative to other loci. Because we only found three individuals with HI between 0.5 and 0.95, we cannot draw conclusions about the behavior of any loci in *F. selysi*-like hybrids. To further characterize this pattern of asymmetric hybridization, we genotyped mtDNA in 40 individuals with HI between 0.05 and 0.95, of which 39 (97%) carried the *F. cinerea* haplotype and one (3%) carried the *F. selysi* haplotype. One hybrid individual failed to amplify. An additional 30 individuals had HI

less than 0.05 (= *F. cinerea*) or greater than 0.95 (= *F. selysi*), and the mtDNA haplotype was perfectly associated with species in these samples (Table S1). When added to a genomic cline analysis, mtDNA was a highly significant outlier, with the *F. selysi* haplotype being underrepresented in hybrids (Fig. S1).

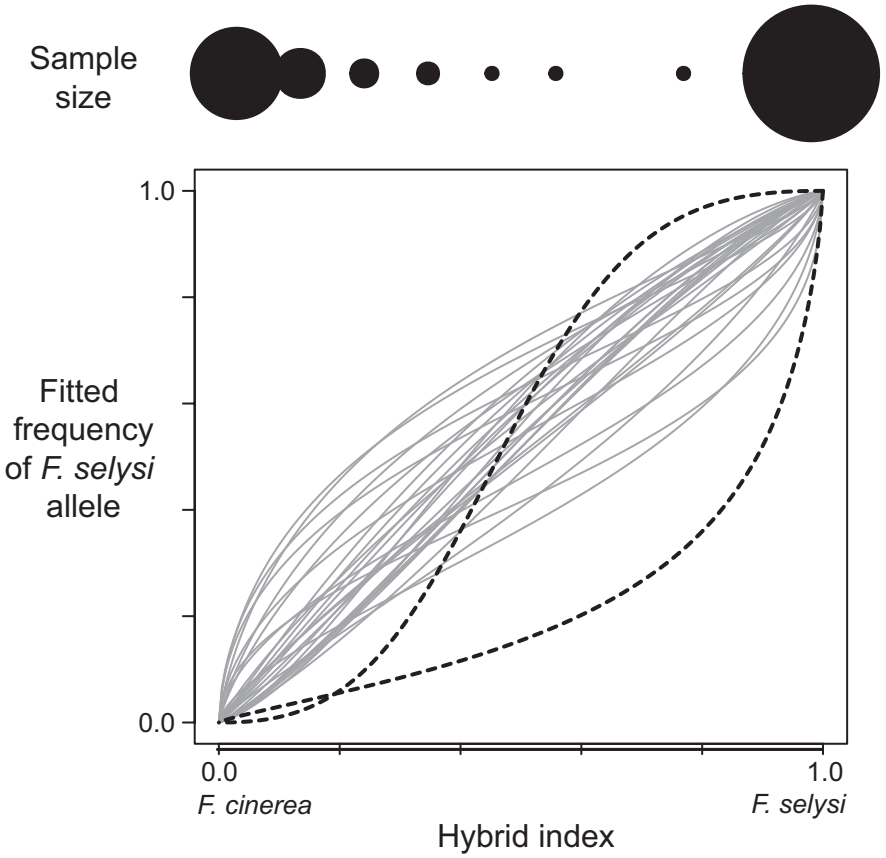

Figure 2. A genomic cline analysis of 30 species-diagnostic SNPs reveals two significant outliers (black dotted lines). Both outlier clines indicate that *F. cinerea*-like hybrids are more likely to harbor *F. cinerea* alleles at these loci. Cline fits may be unreliable for hybrid index values between 0.5 and 0.9, due to low representation of individuals within this HI range (shown in the sample size chart above the panel).

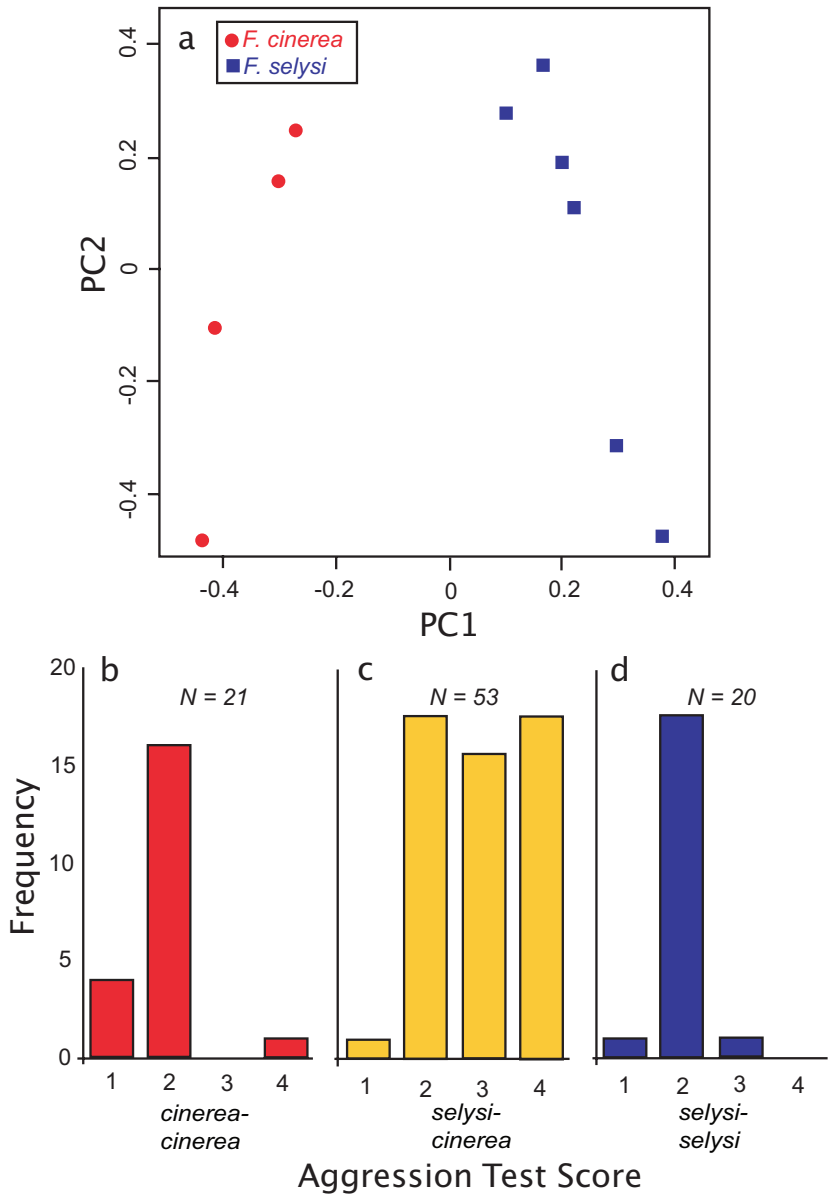

Figure 3. (a) PCA of the cuticular hydrocarbon profiles. Each point represents a colony, while symbols represent different species. The profiles of the two species differ significantly along the first principal component axis, which explains 73% of the total variance. (b, c and d) Aggression scores in pairwise interactions between non-nestmates. The level of aggression was much higher in interspecific trials (c) than in intraspecific trials (b and d).

The social organization of colonies was inferred from the haplotypes at the social supergene, as determined by a STRUCTURE analysis using the 21 loci located in the supergene (Table S2). This analysis revealed that colonies containing hybrid individuals (HI of 0.05-0.95) can be either monogynous or polygnous. Indeed, among 40 hybrids genotyped, 15 individuals had the

monogyne-associated Sm/Sm genotype, while 17 and 8 individuals had polygyne-associated genotypes, Sm/Sp and Sp/Sp, respectively.

Species discrimination: cuticular hydrocarbons and aggression

We found significant differences between the two species in cuticular hydrocarbon profile, as well as an increased level of aggression in interactions between individuals from different species (Fig. 3). *F. cinerea* and *F. selysi* had very different hydrocarbon profiles. The first principal component axis, explaining 73% of the variation, showed no overlap between the two species. There was some variation among colonies within species in the second principal component, which explained an additional 16% of the variance. The PCA revealed no clear differences between workers from different populations within species (not shown). Workers showed strong species discrimination. Biting and acid projection postures occurred frequently between species, but were almost absent within species, and overall there was much more aggression in inter-species interactions than in intra-species interactions (Fig. 3c vs 3b and 3d; permutation test, $p < 0.0001$). We found no difference in the proportion of aggressive interactions initiated by *F. selysi* and *F. cinerea* (binomial test, $p = 0.64$). Moreover, we found no significant difference in aggression between workers from sympatric or allopatric zones, and therefore these were pooled for this analysis (Table S3).

Discussion

We have identified a geographically broad mosaic hybrid zone between *Formica selysi* and *Formica cinerea* along the Rhône river upstream of Lac Léman in Switzerland. Early-generation hybrids (hybrid index between 0.2 and 0.8) are infrequent compared to parental species, and nearly all hybrids are genetically closer to *F. cinerea* than to *F. selysi*. This suggests that there are mechanisms reducing interspecific mating or the success of colonies containing hybrid individuals, and that these reproductive barriers are asymmetric. The presence of

different cuticular hydrocarbon signatures in the two species and their increased interspecific aggression tendencies may be factors maintaining distinct species within this contact zone.

The asymmetry in the distribution of hybrid index (HI), which is biased toward *F. cinerea*, and the fact that 97% of hybrids have the *F. cinerea* mitochondrial haplotype, reveal an intriguing pattern of asymmetrical gene flow with a genome-wide nuclear introgression from *F. selysi* into *F. cinerea* (e.g. While *et al.* 2015). Taken together, the nuclear and mitochondrial data suggest that *F. selysi* queens mated with *F. cinerea* males rarely produce fertile offspring. The majority of hybrid individuals with HI between 0.1 and 0.4 would then emerge through hybrid queens (from a *F. cinerea* queen mated with a *F. selysi* male) mating with *F. cinerea* males, or through *F. cinerea* queens mating with males of hybrid origin. Either way, hybrid sexuals must be produced and (at least occasionally) have some reproductive success in order to explain the distribution of HIs. Overall, this system should provide an ideal opportunity to investigate the relative effects of pre- and post-zygotic isolating mechanisms in two ant species that differ in the direction and degree of their mutual reproductive barrier.

Two nuclear markers do not follow the general pattern of unidirectional gene flow from *F. selysi* to *F. cinerea*. These outliers in the genomic cline analysis show an underrepresentation of *F. selysi* alleles in hybrids, indicating that these loci might be associated with reproductive barriers. We searched the scaffolds containing the two outliers against the *Camponotus floridanus* and *Apis mellifera* genomes. The first outlier is in a region present in multiple copies in *C. floridanus*, with no known genes. The second outlier is located within the gene TRPA5, which belongs to a gene family involved in sensory integration (Matsuura *et al.* 2009). Further investigation is needed to determine why the *F. selysi* alleles at these markers are underrepresented in hybrids compared to the rest of the genome, and whether these loci contribute to species boundaries in this system.

Interestingly, we observe hybrids in both monogynous and polygynous colonies. This rules out a complete barrier to hybridization in either social form. Our genomic analysis reveals that both species share the supergene haplotypes that are associated with social organization in *F. selysi* (Purcell *et al.* 2014b). In our surveys of *F. selysi*, polygynous colonies never produce Sm males or Sm/Sm queens and workers, and monogynous colonies never contain Sp genotypes. Thus, if queens of monogynous origin did not hybridize, we would not encounter hybrid Sm/Sm workers. Similarly, if sexuals of polygynous origin did not hybridize, we would never encounter hybrid Sm/Sp or Sp/Sp workers. We do not yet know, however, whether haplotypes of the social supergene are shared through introgression within the hybrid zone, or whether the divergence of the Sm and Sp haplotypes of the supergene predates the divergence between the two species.

We identified significant differences in the cuticular hydrocarbon profiles of the two species, and higher levels of aggression between workers in interspecific interactions, compared to intraspecific interactions. The increased aggression towards heterospecifics may play a role in speciation, as high levels of aggression between species may result in interspecific exclusion, biased mating, and possibly reduced fitness of hybrid colonies.

How does this system fit into the broader picture of ant hybrid zones? In several well-studied species, hybridization has been coopted to generate genetic caste determination, whereby hybrid offspring are workers, while non-hybrid diploid offspring become new queens (Helms Cahan & Keller 2003; Schwander *et al.* 2007; Leniaud *et al.* 2012). This system results in a different pattern from the one that we observed—hybrid workers would have HI of 0.5, while queens and males would not be hybrids. Pearson (1983) observed an asymmetrical hybrid zone between *Lasius alienus* and *L. niger* in the UK, wherein *L. alienus* queens mated with *L. niger* males produced hybrid workers and males. Thus, only F1 hybrids (workers) were detected,

since haploid males were produced parthenogenetically. In the hybrid zone that we investigate here, the signature of backcrossing in the distribution of hybrid indexes, with the majority of hybrids having HIs between 0.1 and 0.4, shows that hybrid sexuals are produced and can themselves produce viable offspring in the *Formica selysi/cinerea* hybrid zone. The hybrid zone described in this study bears a stronger resemblance to the mosaic hybrid zone between *Solenopsis invicta* and *S. richteri* in North America (Shoemaker *et al.* 1996). Shoemaker *et al.* (1996) suggested that the hybrid zone in *Solenopsis* was likely to be maintained by reduced fitness of hybrid sexuals and/or reduced competitive ability of hybrid colonies. Similar mechanisms may be in place in the *F. selysi/cinerea* hybrid zone if, for example, fertility is reduced or nestmate recognition is disrupted in hybrids.

Beyond ants, mosaic hybrid zones and asymmetric hybridization have been identified in many other systems. Mosaic hybrid zones can arise from species-specific adaptation to patchily distributed habitat types (Harrison 1986; Rand & Harrison 1989), or from stochastic patterns of colonization (Harrison 1986), especially in combination with assortative mating (M'Gonigle & FitzJohn 2009). Asymmetric introgression in hybridizing species is common (e.g. Johnson *et al.* 2015; Kenney & Sweigart 2016; Sardell & Uy 2016), and may result from many causes including hybrid zone movement (Barton & Hewitt 1985; Excoffier *et al.* 2009), patterns of mate choice (Svensson *et al.* 2007), species differences in dispersal (Currat *et al.* 2008), or the genetic architecture of reproductive incompatibilities (Tiffin *et al.* 2001). Future work on patterns of mate choice, ecological differences and genetic differentiation between *F. selysi* and *F. cinerea* will help to elucidate the causes of the mosaic structure and asymmetric introgression that we have identified.

The hybrid zone between *F. selysi* and *F. cinerea* that we describe here provides a promising opportunity to investigate how speciation functions in eusocial insects. Because hybrids are

present, we should be able to tease apart the relative roles of mate choice, reduced fitness or viability in hybrid queens or males, and colony-level incompatibilities. Overall, investigating this and similar systems will provide valuable insights into how speciation works in social organisms.

Acknowledgements

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Supplementary materials

Table S1. Distribution of COI genotypes by hybrid index (Fc for *F. cinerea* and Fs for *F. selysi*). The hybrid index is based on the RAD-based STRUCTURE analysis, with 0 representing "pure" *F. cinerea*, and 1 representing "pure" *F. selysi*.

Individual	HI	COI SNP
Riddes8-w3	0	Fc
Martigny 4-w1	0.001	Fc
Aigle12-w1	0.004	Fc
Aigle11-w1	0.016	Fc
St-Maurice 8-w1	0.041	Fc
Conthey2-w1	0.043	Fc
Aigle3-w1	0.046	Fc
Conthey5-w1	0.053	Fc
Ulrichen4-w1	0.060	Fc
Noville4-w1	0.062	Fc
Noville1-w1	0.071	Fc
Nufenen3-w1	0.071	Fc
Aigle6-w1	0.074	Fc
Conthey6-w1	0.091	Fc
Martigny3-w1	0.092	Fc
Aiglw9-w1	0.093	Fc
Aigle8-w1	0.093	Fc
Nufenen4-w1	0.093	Fc
Noville3-w1	0.094	Fc
Obergestein3-w1	0.100	Fc
Riddes4-w3	0.103	Fc
Aigle4-w1	0.104	Fc
Aigle10-w1	0.109	Fc
Conthey3-w1	0.111	Fc
Obergestein10-w1	0.115	Fc
Fiesch6-w1	0.118	Fc
Obergestein5-w1	0.118	Fc
Conthey1-w1	0.119	Fc
Aigle7-w1	0.130	Fc
Noville2-w1	0.136	Fc
Obergestein2-w1	0.138	Fc
Aigle5-w1	0.140	Fc
Conthey8-w1	0.140	Fc
Conthey7-w1	0.143	Fc
Visp2-w1	0.157	Fc

Conthey9-w1	0.170	Fc
Obrgestein7-w1	0.186	Fc
Visp1-w1	0.209	Fc
Conthey10-w1	0.215	Fc
Riddes3-w1	0.286	Fc
Obergestein6-w1	0.292	Fc
Obergestein1-w1	0.325	Fc
Obergestein8-w1	0.337	Fc
Obergestein9-w1	0.348	Fc
St-Maurice 3-w1	0.522	Fc
Leuk12-w1	0.701	Fs
Riddes6-w3	0.943	Fc
Riddes7-w3	0.989	Fs
Sion3-w1	0.994	Fs
Martigny1-w1	0.999	Fs
Martigny 7-w1	0.999	Fs
Martigny 6-w1	0.999	Fs
St-Maurice9-w1	0.999	Fs
Riddes2-w2	0.999	Fs
Sion6-w1	0.999	Fs
Sion2-w1	0.999	Fs
Martigny 8-w1	0.999	Fs
Martigny 5-w1	1	Fs
St-Maurice10-w1	1	Fs
St-Maurice 7-w1	1	Fs
St-Maurice 6-w1	1	Fs
St-Maurice 5-w1	1	Fs
St-Maurice 2-w1	1	Fs
St-Maurice 1-w1	1	Fs
Riddes11-w3	1	Fs
Riddes5-w3	1	Fs
Sion9-w1	1	Fs
Sion7-w1	1	Fs
Sion5-w1	1	Fs
Sion4-w1	1	Fs

Table S2. Results of STRUCTURE analysis based on 21 SNPs from the social chromosome for each individual in each locality. We obtained three genetic clusters (K = 3), which corresponded to Sp ancestry, and Sm ancestry from either the *F. selysi* or *F. cinerea* background.

Individual	Locality	Position on Map in Fig. 1	F. selysi Sm ancestry	Sp ancestry	F. cinerea Sm ancestry	Inferred social genotype
16	Finges	K	0.978	0.01	0.012	Sm/Sm
171	Finges	K	0.383	0.602	0.015	Sm/Sp
173	Finges	K	0.979	0.01	0.011	Sm/Sm
174	Finges	K	0.008	0.986	0.006	Sp/Sp
181	Finges	K	0.978	0.009	0.013	Sm/Sm
182	Finges	K	0.984	0.008	0.009	Sm/Sm
184	Finges	K	0.969	0.017	0.014	Sm/Sm
186	Finges	K	0.981	0.009	0.01	Sm/Sm
188	Finges	K	0.972	0.016	0.012	Sm/Sm
189	Finges	K	0.979	0.011	0.01	Sm/Sm
194-2P	Finges	K	0.5	0.473	0.028	Sm/Sp
199-2p	Finges	K	0.542	0.436	0.021	Sm/Sp
200-2	Finges	K	0.975	0.013	0.012	Sm/Sm
211-2M	Finges	K	0.978	0.009	0.013	Sm/Sm
212-2	Finges	K	0.976	0.013	0.011	Sm/Sm
215-2	Finges	K	0.969	0.015	0.016	Sm/Sm
216-2	Finges	K	0.975	0.012	0.013	Sm/Sm
228w2	Finges	K	0.977	0.01	0.013	Sm/Sm
230w2	Finges	K	0.975	0.013	0.012	Sm/Sm
233-2	Finges	K	0.529	0.456	0.016	Sm/Sp
234w2	Finges	K	0.534	0.445	0.021	Sm/Sp
238w2	Finges	K	0.458	0.522	0.019	Sm/Sp
240w2	Finges	K	0.977	0.01	0.013	Sm/Sm
243w2	Finges	K	0.978	0.013	0.009	Sm/Sm
245w2	Finges	K	0.539	0.451	0.01	Sm/Sp
246w2	Finges	K	0.968	0.018	0.014	Sm/Sm
247w2	Finges	K	0.455	0.529	0.016	Sm/Sp
42	Finges	K	0.98	0.01	0.01	Sm/Sm
51	Finges	K	0.968	0.021	0.011	Sm/Sm
62	Finges	K	0.973	0.013	0.014	Sm/Sm
69	Finges	K	0.973	0.013	0.015	Sm/Sm
9	Finges	K	0.364	0.619	0.018	Sm/Sp
92	Finges	K	0.976	0.01	0.014	Sm/Sm
Aigle10w2	Aigle	B	0.028	0.489	0.483	Sm/Sp
Aigle11	Aigle	B	0.009	0.985	0.006	Sp/Sp
Aigle12	Aigle	B	0.146	0.458	0.396	Sm/Sp
Aigle3w2	Aigle	B	0.167	0.609	0.224	Sm/Sp
Aigle4w2	Aigle	B	0.021	0.544	0.435	Sm/Sp
Aigle5w2	Aigle	B	0.029	0.525	0.447	Sm/Sp
Aigle6w2	Aigle	B	0.038	0.698	0.264	Sm/Sp
Aigle7w2	Aigle	B	0.03	0.49	0.48	Sm/Sp
Aigle8w2	Aigle	B	0.088	0.889	0.023	Sp/Sp
Aigle9w2	Aigle	B	0.051	0.534	0.415	Sm/Sp
Biel1	Biel	Q	0.012	0.981	0.007	Sp/Sp
Biel2	Biel	Q	0.009	0.005	0.986	Sm/Sm
Biel3	Biel	Q	0.009	0.006	0.985	Sm/Sm
Biel4	Biel	Q	0.024	0.01	0.966	Sm/Sm

Blitzingen1	Blitzingen	P	0.011	0.008	0.981	Sm/Sm
Blitzingen2	Blitzingen	P	0.286	0.022	0.691	Sm/Sm
Blitzingen3	Blitzingen	P	0.013	0.98	0.007	Sp/Sp
Blitzingen4	Blitzingen	P	0.033	0.458	0.509	Sm/Sp
Blitzingen5	Blitzingen	P	0.022	0.009	0.969	Sm/Sm
Blitzingen6	Blitzingen	P	0.013	0.98	0.007	Sp/Sp
Blitzingen7	Blitzingen	P	0.013	0.006	0.981	Sm/Sm
Blitzingen8	Blitzingen	P	0.012	0.981	0.007	Sp/Sp
Conthey1	Conthey	H	0.072	0.879	0.049	Sp/Sp
Conthey10	Conthey	H	0.021	0.545	0.435	Sm/Sp
Conthey2	Conthey	H	0.025	0.963	0.012	Sp/Sp
Conthey3	Conthey	H	0.054	0.923	0.022	Sp/Sp
Conthey5	Conthey	H	0.023	0.964	0.012	Sp/Sp
Conthey6	Conthey	H	0.086	0.889	0.026	Sp/Sp
Conthey7	Conthey	H	0.182	0.795	0.023	Unknown
Conthey8	Conthey	H	0.012	0.982	0.006	Sp/Sp
Conthey9	Conthey	H	0.091	0.013	0.896	Sm/Sm
DE102	Derborence	F	0.555	0.433	0.013	Sm/Sp
DE103	Derborence	F	0.428	0.554	0.018	Sm/Sp
DE107	Derborence	F	0.016	0.976	0.008	Sp/Sp
DE108	Derborence	F	0.59	0.4	0.01	Sm/Sp
DE111	Derborence	F	0.572	0.416	0.012	Sm/Sp
DE112	Derborence	F	0.557	0.431	0.012	Sm/Sp
DE113	Derborence	F	0.461	0.516	0.023	Sm/Sp
DE114	Derborence	F	0.51	0.476	0.014	Sm/Sp
DE43	Derborence	F	0.965	0.018	0.017	Sm/Sm
DE44	Derborence	F	0.976	0.012	0.012	Sm/Sm
DE45	Derborence	F	0.973	0.012	0.016	Sm/Sm
DE46	Derborence	F	0.962	0.016	0.022	Sm/Sm
DE47	Derborence	F	0.971	0.017	0.013	Sm/Sm
DE48	Derborence	F	0.972	0.011	0.016	Sm/Sm
DE49	Derborence	F	0.978	0.013	0.009	Sm/Sm
DE50	Derborence	F	0.973	0.012	0.016	Sm/Sm
DE51	Derborence	F	0.976	0.011	0.013	Sm/Sm
DE67	Derborence	F	0.974	0.015	0.011	Sm/Sm
DE84	Derborence	F	0.979	0.01	0.011	Sm/Sm
DE85	Derborence	F	0.966	0.013	0.021	Sm/Sm
DE86	Derborence	F	0.968	0.017	0.015	Sm/Sm
DE87	Derborence	F	0.981	0.009	0.011	Sm/Sm
DE94	Derborence	F	0.416	0.568	0.016	Sm/Sp
DE95	Derborence	F	0.505	0.481	0.014	Sm/Sp
DE96	Derborence	F	0.454	0.533	0.013	Sm/Sp
DE97	Derborence	F	0.483	0.502	0.015	Sm/Sp
DE98	Derborence	F	0.501	0.482	0.016	Sm/Sp
Fiesch1	Fiesch	O	0.276	0.04	0.684	Sm/Sm
Fiesch10	Fiesch	O	0.968	0.015	0.016	Sm/Sm
Fiesch2	Fiesch	O	0.213	0.056	0.732	Sm/Sm
Fiesch3	Fiesch	O	0.971	0.014	0.015	Sm/Sm
Fiesch4	Fiesch	O	0.974	0.014	0.012	Sm/Sm
Fiesch5	Fiesch	O	0.97	0.015	0.014	Sm/Sm
Fiesch6	Fiesch	O	0.466	0.027	0.507	Sm/Sm
Fiesch7	Fiesch	O	0.013	0.007	0.98	Sm/Sm
Fiesch9	Fiesch	O	0.371	0.015	0.614	Sm/Sm
Goms1	Goms	R	0.012	0.982	0.006	Sp/Sp
Goms2	Goms	R	0.011	0.979	0.01	Sp/Sp
Goms3	Goms	R	0.127	0.56	0.313	Sm/Sp
Goms4	Goms	R	0.031	0.458	0.511	Sm/Sp
LH1	Les Hauderes	J	0.97	0.021	0.009	Sm/Sm

LH10	Les Hauderes	J	0.98	0.01	0.01	Sm/Sm
LH11	Les Hauderes	J	0.153	0.831	0.016	Sp/Sp
LH12	Les Hauderes	J	0.015	0.979	0.006	Sp/Sp
LH13	Les Hauderes	J	0.267	0.724	0.009	Unknown
LH14	Les Hauderes	J	0.492	0.495	0.013	Sm/Sp
LH2	Les Hauderes	J	0.983	0.009	0.009	Sm/Sm
LH3	Les Hauderes	J	0.976	0.014	0.009	Sm/Sm
LH4	Les Hauderes	J	0.979	0.013	0.008	Sm/Sm
LH5	Les Hauderes	J	0.017	0.978	0.006	Sp/Sp
LH6	Les Hauderes	J	0.98	0.01	0.01	Sm/Sm
LH7	Les Hauderes	J	0.981	0.009	0.009	Sm/Sm
LH8	Les Hauderes	J	0.012	0.981	0.007	Sp/Sp
LH9	Les Hauderes	J	0.012	0.982	0.006	Sp/Sp
Leuk1	Leuk	L	0.978	0.012	0.01	Sm/Sm
Leuk10	Leuk	L	0.966	0.022	0.012	Sm/Sm
Leuk11	Leuk	L	0.01	0.007	0.983	Sm/Sm
Leuk12	Leuk	L	0.026	0.965	0.009	Sp/Sp
Leuk13	Leuk	L	0.409	0.58	0.011	Sm/Sp
Leuk14	Leuk	L	0.014	0.98	0.006	Sp/Sp
Leuk15	Leuk	L	0.98	0.009	0.011	Sm/Sm
Leuk16	Leuk	L	0.969	0.02	0.011	Sm/Sm
Leuk2	Leuk	L	0.968	0.017	0.015	Sm/Sm
Leuk3	Leuk	L	0.979	0.009	0.011	Sm/Sm
Leuk4	Leuk	L	0.979	0.01	0.011	Sm/Sm
Leuk5	Leuk	L	0.977	0.014	0.01	Sm/Sm
Leuk6	Leuk	L	0.972	0.015	0.012	Sm/Sm
Leuk7	Leuk	L	0.964	0.023	0.012	Sm/Sm
Leuk8	Leuk	L	0.973	0.017	0.009	Sm/Sm
Leuk9	Leuk	L	0.979	0.01	0.011	Sm/Sm
Martigny1	Martigny	D	0.973	0.016	0.012	Sm/Sm
Martigny10	Martigny	D	0.148	0.62	0.233	Sm/Sp
Martigny2	Martigny	D	0.961	0.026	0.013	Sm/Sm
Martigny3	Martigny	D	0.043	0.444	0.513	Sm/Sp
Martigny4	Martigny	D	0.032	0.511	0.457	Sm/Sp
Martigny5	Martigny	D	0.353	0.51	0.136	Sm/Sp
Martigny6	Martigny	D	0.193	0.648	0.159	Sm/Sp
Martigny7	Martigny	D	0.57	0.42	0.01	Sm/Sp
Martigny8	Martigny	D	0.558	0.427	0.015	Sm/Sp
Naters1	Naters	N	0.026	0.431	0.542	Sm/Sp
Naters4	Naters	N	0.011	0.983	0.006	Sp/Sp
Noville1w2	Noville	A	0.053	0.395	0.552	Sm/Sp
Noville2w2	Noville	A	0.388	0.017	0.595	Sm/Sm
Noville3w2	Noville	A	0.017	0.975	0.008	Sp/Sp
Noville4w2	Noville	A	0.05	0.525	0.425	Sm/Sp
NufenenO_1	Nufenen	U	0.227	0.025	0.748	Sm/Sm
NufenenO_10	Nufenen	U	0.025	0.01	0.965	Sm/Sm
NufenenO_3	Nufenen	U	0.085	0.01	0.905	Sm/Sm
NufenenO_4	Nufenen	U	0.109	0.008	0.883	Sm/Sm
NufenenO_5	Nufenen	U	0.041	0.051	0.908	Sm/Sm
NufenenO_6	Nufenen	U	0.172	0.183	0.645	Sm/Sm
NufenenO_7	Nufenen	U	0.041	0.011	0.948	Sm/Sm
NufenenO_8	Nufenen	U	0.107	0.169	0.724	Sm/Sm
NufenenO_9	Nufenen	U	0.022	0.523	0.455	Sm/Sp
Obergestein	Obergestein	T	0.028	0.475	0.498	Sm/Sp
Obergestein	Obergestein	T	0.029	0.507	0.464	Sm/Sp
Obergestein	Obergestein	T	0.03	0.452	0.518	Sm/Sp
Obergestein	Obergestein	T	0.028	0.468	0.504	Sm/Sp
Obergestein	Obergestein	T	0.037	0.11	0.854	Sm/Sm

Obergestein	Obergestein	T	0.03	0.143	0.826	Sm/Sm
Obergestein	Obergestein	T	0.045	0.354	0.602	Sm/Sp
Obergestein	Obergestein	T	0.42	0.021	0.559	Sm/Sm
Obergestein	Obergestein	T	0.035	0.507	0.457	Sm/Sp
Obergestein	Obergestein	T	0.068	0.526	0.406	Sm/Sp
Orsieres1	Orsieres	E	0.338	0.65	0.012	Sm/Sp
Orsieres2	Orsieres	E	0.611	0.378	0.012	Sm/Sp
Orsieres3	Orsieres	E	0.189	0.789	0.022	Unknown
Orsieres4	Orsieres	E	0.39	0.599	0.011	Sm/Sp
Orsieres5	Orsieres	E	0.583	0.381	0.035	Sm/Sp
Orsieres6	Orsieres	E	0.411	0.496	0.093	Sm/Sp
Riddes1	Riddes	G	0.468	0.519	0.013	Sm/Sp
Riddes11	Riddes	G	0.449	0.532	0.019	Sm/Sp
Riddes2	Riddes	G	0.977	0.011	0.012	Sm/Sm
Riddes3	Riddes	G	0.048	0.013	0.938	Sm/Sm
Riddes4	Riddes	G	0.011	0.006	0.983	Sm/Sm
Riddes5	Riddes	G	0.507	0.478	0.015	Sm/Sp
Riddes6	Riddes	G	0.12	0.538	0.342	Sm/Sp
Riddes7	Riddes	G	0.021	0.973	0.006	Sp/Sp
Riddes8	Riddes	G	0.02	0.024	0.956	Sm/Sm
Riddes9	Riddes	G	0.048	0.54	0.412	Sm/Sp
Sion1	Sion	I	0.428	0.554	0.018	Sm/Sp
Sion10	Sion	I	0.479	0.504	0.017	Sm/Sp
Sion2	Sion	I	0.974	0.011	0.015	Sm/Sm
Sion3	Sion	I	0.024	0.969	0.007	Sp/Sp
Sion4	Sion	I	0.972	0.019	0.009	Sm/Sm
Sion5	Sion	I	0.976	0.013	0.012	Sm/Sm
Sion6	Sion	I	0.975	0.015	0.01	Sm/Sm
Sion7	Sion	I	0.97	0.011	0.019	Sm/Sm
Sion8	Sion	I	0.548	0.44	0.012	Sm/Sp
Sion9	Sion	I	0.55	0.439	0.012	Sm/Sp
St-Maurice1	St. Maurice	C	0.503	0.486	0.011	Sm/Sp
St-Maurice2	St. Maurice	C	0.977	0.011	0.012	Sm/Sm
St-Maurice3	St. Maurice	C	0.45	0.011	0.539	Sm/Sm
St-Maurice4	St. Maurice	C	0.011	0.006	0.984	Sm/Sm
St-Maurice5	St. Maurice	C	0.531	0.458	0.011	Sm/Sp
St-Maurice6	St. Maurice	C	0.517	0.472	0.011	Sm/Sp
St-Maurice7	St. Maurice	C	0.536	0.451	0.013	Sm/Sp
St-Maurice8	St. Maurice	C	0.465	0.523	0.012	Sm/Sp
St-Maurice9	St. Maurice	C	0.4	0.587	0.012	Sm/Sp
Ulrichen1	Ulrichen	S	0.309	0.026	0.665	Sm/Sm
Ulrichen2	Ulrichen	S	0.105	0.056	0.839	Sm/Sm
Ulrichen3	Ulrichen	S	0.115	0.017	0.868	Sm/Sm
Ulrichen4	Ulrichen	S	0.03	0.01	0.959	Sm/Sm
Visp1	Visp	M	0.073	0.355	0.572	Sm/Sp
Visp2	Visp	M	0.157	0.642	0.2	Sm/Sp
Visp3	Visp	M	0.011	0.981	0.008	Sp/Sp
Visp4	Visp	M	0.031	0.388	0.581	Sm/Sp

Table S3. Aggression trial scores for intra- and interspecific pairwise aggression trials with workers from the same population (= sympatric) or different populations (= allopatric).

Aggression Score	Sympatric	selysi x selysi	cinerea x cinerea	selysi x cinerea
1		1	1	0
2		10	10	5
3		0	0	2
4		0	1	4
	Allopatric			
1		0	3	1
2		8	6	13
3		1	0	14
4		0	0	14

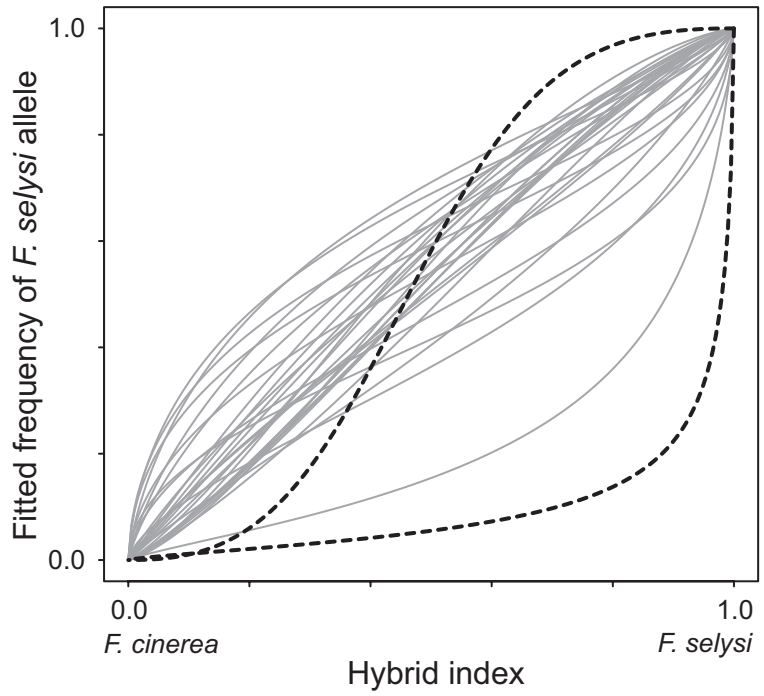

Figure S1. Genomic cline analysis of 30 species diagnostic nuclear SNPs and one mtDNA SNP. Significant outliers, one nuclear marker and the mtDNA marker, are shown with dotted black lines.

BOX 1
PCR-RFLP Protocol for Genotyping Markers on the Social Chromosome

Primer Sequences

Primer set 1: Fs3999; *HinfI cuts Sm allele, not Sp allele*

Fs3999-F1	ACCGGCTTTCAGTCTGACA
Fs3999-R1	TGCGCTTCCCTTATATAATGGTG
Fs3999-F2	ATGTGTCATTCAGCGTCGC
Fs3999-R2	CAAAGATTTCTCATTCTCTGCGA

Primer set 2: Fs1398; *HinfI cuts Sm allele, not Sp allele*

Fs1398-F1	AAACTTCGACTGAGCACGC
Fs1398-R1	ATTGACCACGTTGTTGCCC
Fs1398-F2	CTGCGTCGTAGTAGGGCTT
Fs1398-R2	GGCATCGTTGTGCTGAGAG

PCR 1, Master Mix

REAGENT	1X VOLUME	100X VOLUME
WATER	6.84	684
10X BUFFER	1	100
DNTP (25MM)	0.08	8
TAQ	0.08	8
PREMIXED PRIMER 1398-1	0.5	50
PREMIXED PRIMER 3999-1	0.5	50
TOTAL	9µL	900µL

Add 1 μL DNA for each reaction

PCR conditions:

94° C for 3 min; 30 cycles of: 94° C for 30s, 54.4° C for 30s, 72° C for 40s; final extension at 72° C for 3 min.

Dilute the product of PCR 1 10x (so add 90 μL of water to each well), mix

PCR 2a//2b, Master Mix (PCRs should be run separately for each primer set)**

REAGENT	1X VOLUME	100X VOLUME
WATER	6.84	684
10X BUFFER	1	100
DNTP (25MM)	0.08	8
TAQ	0.08	8
PREMIXED PRIMER 1398-2//3999-2	1	100
TOTAL	9µL	900µL

Add 1 μL diluted PCR product for each reaction

Repeat for PCR 2b (substitute premixed primer 3999-2)

PCR conditions:

94° C for 3 min; 30 cycles of: 94° C for 30s, 54.4° C for 30s, 72° C for 40s; final extension at 72° C for 3 min.

Restriction Digest, Master Mix

REAGENT	1X VOLUME	100X VOLUME
WATER	5.3	530
BUFFER CUTSMART	0.6	60
HINFI	0.1	10
TOTAL	6µL	600µL

Add 3 μL PCR product for each reaction. Incubate at 37° C for at least 60 minutes.

Taxonomy of F. selysi and F. cinerea

The present study, based on many nuclear SNPs, revealed a deeper genomic divergence between the *Serviformica* lineages than suspected in a previous analysis based on microsatellites and mtDNA COI sequences (Purcell *et al.* 2015). Assuming a mitochondrial divergence rate of ~2% per million years as observed in other invertebrates (e.g. Wilke *et al.* 2009), the divergence time between Swiss *F. selysi* and *F. cinerea* is roughly 0.15 million years. We suggest that this low divergence is likely to result from mitochondrial capture (Toews & Brelsford 2012). Theory predicts a high rate of mitochondrial introgression in haplodiploids (Patten *et al.* 2015) and mitochondrial capture has probably occurred in other *Formica* species (Goropashnaya *et al.* 2004). However, we cannot rule out recent and rapid speciation. We note that the reassessment of previous samples from the Rhône, Rhine, and Po drainages (here assessed as *F. selysi, F. selysi* and *F. cinerea,* respectively) does not undermine our previous conclusion that the frequency of monogynous colonies increases at higher elevations in three independent genetic lineages (Purcell *et al.* 2015).

Chapter 2

Species recognition limits mating between hybridizing ant species

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Authors' contributions

SZ, PB, JP, AB and MC planned and designed the study. SZ, PB and TOH performed field sampling and laboratory work with the help of AA, JP and AB. SZ and AA performed the mating experiment and the hybrid viability experiment. PB and TOH performed and analyzed the aggression tests. PB and SZ performed CHC extraction. GBR, DS and PB performed the CHC analysis. SZ and PB analyzed the data. SZ, PB and MC wrote the manuscript, with contribution of all the authors. All authors read and approved the manuscript.

Abstract

Identifying mechanisms limiting hybridization is a central goal of speciation research. Here, we studied the impact of pre-mating and post-mating barriers on hybridization between two ant species, *Formica selysi* and *Formica cinerea*. These species hybridize in the Rhône valley in Switzerland, leading to the formation of a mosaic hybrid zone showing moderate levels of introgression, mostly from *F. selysi* into *F. cinerea*. There was no sign of temporal isolation between the two species in the production of queens and males. With choice experiments, we showed that queens and males strongly prefer to mate with conspecifics. We did not detect post-mating barriers caused by genetic incompatibilities. Specifically, hybrids of all sexes and castes were found in the field and F1 hybrid workers did not show reduced viability compared to non-hybrid workers. To gain insights into the proximate causes of assortative mate choice, we staged dyadic encounters between workers and analyzed their cuticular hydrocarbons. The cuticular hydrocarbons of workers differed strongly between species, and workers of both species showed good species recognition abilities. We propose that the well-developed recognition system of ants facilitates the establishment of pre-mating barriers to hybridization, even in the absence of strong hybridization costs.

Introduction

Hybridization and gene flow between species play key roles in fundamental evolutionary processes such as adaptation and speciation. The widespread application of genome sequencing has led to the realization that hybridization is more common than previously thought (Mallet 2005, 2007; Ellstrand & Rieseberg 2016). By bringing together independently evolving genomes, hybridization often negatively affects the fitness of individuals, a phenomenon known as 'hybrid breakdown' (Coyne 1998; Burke & Arnold 2001; Abbott *et al.* 2013). Such costs might play a crucial role in the early evolution and later maintenance of species, by selecting for mechanisms limiting interspecific gene flow.

Hybridization can be limited through multiple reproductive isolating barriers occurring before or after mating (Coyne & Orr 2004). Pre-mating barriers to hybridization include spatial isolation, temporal isolation, and mate choice. Post-mating barriers to introgression comprise hybrid inviability and hybrid sterility. The various mechanisms influence each other in a feedback loop (i.e. reinforcement; Servedio & Noor 2003; Coyne & Orr 2004). In particular, low hybrid fitness selects for assortative mating (Coyne 1998; Burke & Arnold 2001; Coyne & Orr 2004; Chatfield *et al.* 2010; Abbott *et al.* 2013; Shizuka & Hudson 2020). However, species recognition and assortative mating can also evolve independently of hybridization costs, due to drift or local adaptation (Hollander *et al.* 2005), or as a by-product of sexual or kin selection (Gleason & Ritchie 1998; Servedio 2016). Determining the impact and causal relationship of pre- and post-mating isolation mechanisms on hybridization patterns is important to understanding the evolutionary processes limiting hybridization and leading to speciation (Irwin 2020).

The speciation process has received surprisingly little attention in the social Hymenoptera. Yet, social Hymenoptera present several characteristics that make them valuable models for investigating

hybridization patterns (Seifert 1999; Feldhaar *et al.* 2008; Kulmuni *et al.* 2010; Beresford *et al.* 2017). Because of their male-haploid female-diploid sex determination system, males are expected to suffer higher fitness consequences of hybridization, as all introgressed alleles are exposed to selection. This fitness asymmetry can lead to hybrid zones composed of hybrid females and non-hybrid males (Kulmuni & Pamilo 2014). Furthermore, in many species females predominantly mate with a single male at the beginning of their adult lives. This lifelong commitment between partners exposes them to large hybridization costs, as potential genetic incompatibilities between mates cannot be mitigated by remating (Feldhaar *et al.* 2008). Fertile hybrid queens and males are generally rare in the social Hymenoptera, which suggests that hybridization costs are high (Kulmuni *et al.* 2010; Feldhaar *et al.* 2008). High hybridization costs may favor the evolution of effective pre-mating barriers. Yet, hybridization is common in several ant lineages (Feldhaar *et al.* 2008) and in some systems it results in hybridogenesis, whereby workers are systematically produced by hybridization between two lineages (Lavanchy & Schwander 2019). In view of this diversity of outcomes, the relationship between hybridization costs, species recognition and reproductive isolation deserves further investigation.

The recognition system of social Hymenoptera could facilitate the evolution of pre-mating barriers. Because insect societies are vulnerable to exploitation by non-kin, they have developed effective recognition systems that allow them to discriminate and reject non-nestmate intruders. This recognition system is based on chemicals, mostly hydrocarbons, present on the insect cuticles and forming a colony-specific odor (d'Ettorre & Lenoir 2010; Bos & d'Ettorre 2012). The behavioral response of individuals, affiliative or agonistic, depends on the similarity of their cuticular hydrocarbon (=CHC) profiles (Guerrieri & D'Ettorre 2008). CHCs have a strong genetic component, so that conspecifics tend to have similar CHC profiles (Guerrieri & D'Ettorre 2008; Gleason *et al.* 2009; Dembeck *et al.* 2015). Social insect workers typically

behave more aggressively toward individuals from other species than toward conspecifics (Lai *et al.* 2015). Nestmate recognition mechanisms could hence facilitate the evolution of species discrimination and assortative mating, even when hybridization costs are low or absent (Drescher *et al.* 2010; Xue *et al.* 2018). Furthermore, the CHC profiles of hybrids are likely to influence the behavioral interactions of hybrids with members of their parent species. Investigating the CHC profiles of hybrids and the mutual behavior of hybrids and parental species can thus help to explain the dynamics of hybrid zones in social insects.

The discovery of a mosaic hybrid zone between the ant species *Formica selysi* and *Formica cinerea* (Purcell *et al.* 2016) set an ideal foundation for the study of barriers to hybridization in ants. *F. cinerea* is broadly distributed across Europe, whereas *F. selysi* is present in valleys of the Alps and the Pyrenees. Both species occupy sparsely vegetated, sunny, sandy areas. *F. selysi* is particularly abundant near streams and rivers. Hybridization was reported along the Rhône valley in Switzerland (Purcell *et al.* 2016). Interestingly, hybrids were relatively rare, amounting to 20% of the workers genotyped. Most hybrid workers had a genetic background skewed toward *F. cinerea*. These genomic data suggest that some mechanisms restrict gene flow between species, but also that hybrids are fertile and mainly backcross with *F. cinerea* (Purcell *et al.* 2016). Preliminary assessment of the CHC profiles and behavior of workers from the two species also suggested that species recognition mechanisms might play a role in this asymmetric hybridization (Purcell *et al.* 2016), possibly helping stabilize the mosaic hybrid zone (M'Gonigle & FitzJohn 2010). This prompted us to investigate the temporal, behavioral and genetic barriers to hybridization between the two species and to further study the discrimination ability and recognition cues of hybrid and pure species workers.

To better understand the maintenance and dynamics of this hybrid zone, we investigated two potential pre-mating barriers, temporal segregation and assortative mate preference, and one potential post-mating barrier, the reduced viability of hybrid offspring caused by genetic

incompatibilities. We show that species recognition and assortative mate preference occur in both species and discuss how asymmetries in discrimination might limit and bias gene flow between the two species.

Materials and Methods

General experimental approach, sampling and genotyping

We assessed temporal isolation between *F. selysi* and *F. cinerea* by monitoring the timing of production of winged queens and males. We then staged controlled mate choice experiments to examine whether queens and males preferentially mated with partners of their own species. To study genetic incompatibilities between species, we monitored brood production by queens mated with conspecifics or heterospecifics. In addition, we checked whether viable hybrid workers, queens and males occurred in the field. Finally, we examined whether workers behaviorally discriminated conspecifics, hybrids and heterospecifics, and studied the cuticular hydrocarbons (=CHCs) likely involved in species recognition.

Field sampling and monitoring took place in 10 populations from central Valais, Switzerland. These populations harbor pure *F. selysi*, pure *F. cinerea* and/or hybrid individuals, in varying proportions (Purcell et al. 2016). We sampled winged queens, males and workers in June and July 2014, 2015, 2016 and 2018. We collected workers for discrimination tests and CHC analysis in October, 2017.

We identified the species and hybrids by genotyping diagnostic SNPs of at least two workers per colony. DNA was extracted from one leg crushed in 100µl of ddH_20 with 10% Chelex© and 5µl of proteinase K (Qiagen, 20mg/ml), incubated at 55°C for 40min, followed by 100°C for 20mn. With a PCR-RFLP assay, we genotyped one mitochondrial and three nuclear SNPs presenting fixed differences between *F. selysi* and *F. cinerea* (Purcell et al. 2016). Individuals

were classified as hybrids when they were heterozygous at one or more SNPs or had a combination of homozygous SNPs specific to *F. selysi* and *F. cinerea*.

Temporal isolation

To assess whether the timing of queen and male production constitutes a pre-mating barrier to hybridization, we monitored the production of winged queens (i.e. unmated females destined to become queens) and males in 36 colonies of pure *F. selysi* and 16 colonies of pure *F. cinerea*, as inferred by genotyping three workers per colony. The colonies were located in three populations harboring both species (Riddes, Saillon and Branson; Purcell *et al.* 2016). We visited each colony on a weekly basis, in June and July 2014, and recorded the presence or absence of winged queens or males inside the colony.

Mate choice

We sampled winged unmated queens, males and workers from 81 colonies in 10 populations (Purcell *et al.* 2016) during summer 2014, 2015 and 2016. Colony fragments were transferred to plastic boxes (15.5 × 13.5 × 5.5cm) lined with fluon and containing a glass tube (length = 16cm; ø = 5mm) $^1/_3$ filled with water. They were maintained in the laboratory in a 12:12 dark:light cycle, at 24°C and under a relative humidity of 50%. The ants were provided with water and sugar-egg jelly *ad libitum*. We kept the unmated queens and males in separate plastic boxes, to prevent intranidal mating. We genotyped three workers per colony, which allowed us to identify 30 pure *F. selysi*, 15 pure *F. cinerea* and 5 hybrid colonies that produced queens and/or males. We retained the pure *F. selysi* and pure *F. cinerea* colonies for mate choice and genetic incompatibility experiments.

To assess whether queens and males prefer to mate with partners of their own species, we performed mate choice experiments in controlled conditions, following the procedure described in Avril *et al.* (2019). In each assay, one unmated female (queen), either *F. cinerea* or *F. selysi*,

was placed with four color-marked unmated males, two per species, in a mating arena consisting of a box covered by a net (35 x 22 x 15 cm). The female and the males originated from different colonies and, whenever possible, from different populations. The observers were kept blind with respect to the species involved. The mating boxes were placed outdoors, on sunny mornings. We observed the queens and males for up to 120 minutes and collected all mating pairs. We isolated the mated queens in glass tubes $^1/_3$ filled with water.

Genetic incompatibilities between species

To test for genetic incompatibilities between species, we monitored survival and brood production of *F. selysi* and *F. cinerea* queens mated either to males of their own species or to males of the other species. The glass tubes containing the mated queens were covered with foil and placed in the dark to mimic natural conditions of independent colony founding. We monitored each queen individually two to four times a week during six consecutive weeks, recording i) whether the queens were alive, ii) whether they produced a brood and iii) their number of offspring. We included in this experiment all queens that mated in the mate choice experiment, plus additional queens that mated without choice (i.e, were presented to males of only one species, in the same experimental conditions; Avril *et al.* 2019). Details on queen samples are given in Table S1.

We also assessed if genetic incompatibilities affected the production of hybrid winged queens and males by monitoring their production in hybrid field colonies (determined via genotyping of two workers per colony, see above). In June and July, over three years, we visited once or twice hybrid colonies from three populations and recorded the number of winged queens or males present (Table S2; Purcell *et al.* 2016). We genotyped most of the winged queens and males to confirm their hybrid genetic background.

Species recognition: dyadic encounters

We collected workers from the Branson population, which contains pure *F. selysi*, pure *F. cinerea* and hybrid colonies. We genotyped three workers per colony and retained for the experiments three pure *F. selysi* colonies, six pure *F. cinerea* colonies and eight hybrid colonies. We housed the nestmate workers in separate plastic boxes (15.5 × 13.5 × 5.5cm) lined with fluon and containing a glass tube (length = 16cm; ø = 5mm) $^1/_3$ filled with water. The workers were maintained at 25°C, with a humidity level of 70%, in a 12:12 h light:dark cycle. They were provided with sugar-egg jelly twice a week.

To investigate whether individuals recognize and behaviorally discriminate conspecifics, heterospecifics, and hybrids, we performed dyadic encounters. We tested the six following dyads of workers: *F. cinerea* vs. *F. cinerea* (n = 23), *F. selysi* vs. *F. selysi* (n = 20), Hybrid vs. Hybrid (n = 16), *F. cinerea* vs. *F. selysi* (n = 33), Hybrid vs. *F. cinerea* (n = 31), Hybrid vs. *F. selysi* (n = 27). All tested workers within dyads were non-nestmates.

Workers were paint-marked 48h before the assays, using color combinations allowing for individual identification. The dyadic encounters took place in a neutral arena consisting of a 6cm Petri dish side-lined with fluon, with a filter paper on the bottom. For each assay, two workers were transferred to separate compartments of the neutral arena. After one minute, the partitions were removed to allow workers to interact freely. We video-recorded the behavior of workers for three minutes. The tested workers were freeze-killed after the assay, stored in glass vials at -20°C and the filter paper was replaced to remove odors. The order of assays was randomized among the six types of dyadic encounters.

We measured the occurrence and duration of each behavior for each worker involved in a dyadic encounter with the software BORIS v.5.1.0 (Friard & Gamba 2016). The scorer of the videos was kept blind to the species of the tested ants. We calculated an aggression index (AI)

based on the following scores for each behavior (adapted from Hefetz *et al.* 1996; Errard & Hefetz 1997): 0, antennation (= neutral interaction); 1, mandible opening (= threat); 2, biting (= moderately aggressive interaction); 3, biting with acid spreading (= highly aggressive interaction). The overall aggression exhibited by each worker (AI) was calculated as follows:

$$AI = \frac{\sum_{i=1}^{n} AIi * ti}{T}$$

where AI_i represents the score of the interaction i, t_i, the duration of each interaction and T, the total interaction time, defined as the sum of durations of all interactions.

Species recognition: GCMS analysis of cuticular hydrocarbons

To get insights into the cues involved in species recognition, we compared the cuticular hydrocarbons of pure species and hybrid workers. We analysed the hydrocarbon profiles from three *F. selysi* colonies, six *F. cinerea* colonies and eight hybrid colonies, respectively. The cuticular compounds were extracted by immersing three workers in 320 µL of hexane (99% pure) for 15 minutes (two replicates per colony). The solvent extract was transferred to a new vial, where it evaporated. Each extract was then dissolved again in 30 µL of hexane, complemented with 10 ng/µL of eicosane (nC$_{20}$; not present in *F. selysi*), which served as internal standard. Two µL of each extract were injected into an Agilent gas chromatograph tandem mass spectrometer (GC-MSMS Agilent 7010, USA) equipped with a Agilent 19091S-433UI HP5-MS column. The carrier gas (helium) flow rate was set at 3 mL/min. Injection temperature was set to 250°C in splitless mode. The temperature ramp was set at 70°C and increased to 300°C at 3°C/min, then maintained at 300°C for 3 min (total run time: 67.67 min). The analysis was carried out in a full scan acquisition mode (50 to 500 amu).

Peak areas were integrated with OPENChrome software version 1.4.0 and the chromatograms were aligned with the package GcalignR (Ottensmann *et al.* 2018). After manual curation of

the dataset to improve alignment, potential contaminants were excluded by removing all peaks whose relative abundance amounted to less than 0.2% in all species. The relative abundance of the 75 peaks retained for analysis was then re-calculated.

Statistical analysis

All analyses were conducted using R v3.4.4. Models were tested using the "glmmTMB" package (Brooks *et al.* 2017) and regression assumptions were evaluated using diagnostic plots with the package DHARMa (Hartig 2016). Non-significant interaction terms were removed from models. All post-hoc analyses were adjusted for multiple comparisons using FDR (False Discovery Rate) corrections.

Temporal isolation

To assess whether *F. selysi* and *F. cinerea* colonies differed in the timing of production of winged females and males, we performed a custom bootstrap test to compare the temporal overlap between species to a null distribution. To obtain the null distribution, we first calculated the period of time in which winged females or males were observed for each colony. We then randomly allocated each colony to one or the other species and calculated the mean overlapping period between the two groups, repeating this process 10,000 times. We finally compared this null distribution to the observed overlap value.

Mate choice

To assess whether queens preferentially mated with males of their own species, we used a Generalized Linear Mixed Model (GLMM) with binomial error distribution. For each queen, we included the outcome of the trial with each of the four males (=if the queen mated or not) as response variable and the species of the queen, whether the male belonged to the same species or to the other species, and the interaction of these factors as fixed factors. To control

for potential population effects on mating, we also included as fixed factor whether the male and queen originated from the same or from a different population. To account for the non-independence of observations, we included as random factors the trial id, the colony of the queen and the colony of the male.

Genetic incompatibilities between species

To detect potential genetic incompatibilities between species, we analysed the reproductive success of queens using GLMMs. Queens included in this analysis were *F. selysi* or *F. cinerea* queens mated to either *F. selysi* or *F. cinerea* males. Using a model with binomial error distribution, we assessed the probability that queens successfully produced a brood, considering that queens failed when they died or did not produce an offspring before the end of the experiment. We then tested if the queens that successfully produced a brood differed in the number of offspring they produced, using a model with Gaussian error distribution. The queen species, her mate species and the interaction of these factors were included as fixed factors. The year of the experiment and the colonies of origin of the male and queen were included as random factors.

Dyadic encounters

We compared the aggression indexes (AIs) of workers with GLMMs, using a Tweedie error distribution. We fitted one model per focal species. We included the species of the non-focal worker as a fixed factor. To account for the non-independence of observations, we included the colony of origin of the focal worker and the trial id as random factors. All assays in which workers interacted at least one time were included in these analyses.

Chemical analysis

To test for overall differences between the cuticular hydrocarbon profiles of the two pure species and their hybrid, we used distance-based Permutational Multivariate Analysis of Variance (PERMANOVA). We averaged the chemical profiles of the two replicates per colony and fitted a linear model to the Bray-Curtis distances between the averaged chemical profiles (computed from relative abundances of peaks). We performed 10,000 permutations, with the package "adonis".

Results

Temporal isolation

We found no evidence that temporal isolation constitutes a pre-mating barrier to hybridization. Overall, *F. selysi* and *F. cinerea* colonies did not differ significantly in their timing of production of females or males (Bootstrap test; $p = 0.95$; Figure S1). Winged females or males were found inside 38.9% (14/36) and 56.3% (9/16) of the monitored colonies of *F. selysi* and *F. cinerea*, respectively.

Mate choice

Mating was mostly assortative (Figure 1). In mate choice experiments, queens were significantly more likely to mate with conspecific males than with heterospecific males (Estimate = -1.94, SE = 0.47, z = -4.08, $p < 0.0001$), irrespective of the queen species (Interaction: $\chi^2 = 0.41$, $p = 0.52$). *F. selysi* queens mated with *F. selysi* males in 87% (26/30) of the mating events, whereas *F. cinerea* queens mated with *F. cinerea* males in 80% (12/15) of the mating events. Whether the queens and the males originated from the same population did not affect the probability of mating (Estimate = -0.06, SE = 0.38, z = -0.17, $p = 0.87$). *F. selysi*

and *F. cinerea* queens had similar mating success (Estimate = -0.04, SE = 0.37, z = -0.1, p = 0.92).

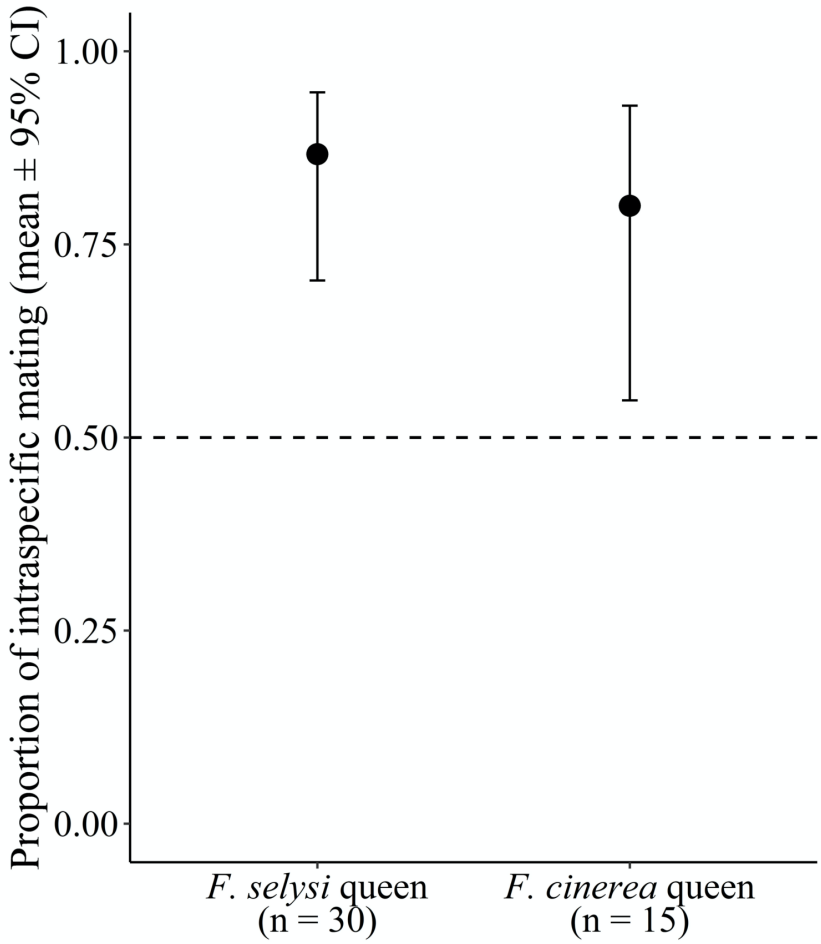

Figure 1. Mate choice of *F. selysi* and *F. cinerea* queens. The dashed line illustrates the expected proportion of intra-specific mating under random mating. n = number of successful mating trials.

Genetic incompatibilities between species

We did not detect post-mating genetic incompatibilities between *F. selysi* and *F. cinerea*. Queens of each species did not differ significantly in their probability of surviving and producing a brood (Estimate = 0.29, SE = 0.54, z = 0.53, p = 0.59; Figure S2A), nor in the number of workers produced (Estimate = -0.48, SE = 1.02, z = -0.47, p = 0.64; Figure S2B).

The species of the queen's mate did not affect the queen's probability of producing a brood (Estimate = -0.27, SE = 0.64, z = -0.42, p = 0.67; Figure S2A) nor the number of offspring produced (Estimate = 0.79, SE = 0.83, z = 0.94, p = 0.35; Figure S2B). More importantly, interspecific crosses did not show signs of genetic incompatibilities, as there was no significant interaction between the queen species and her mate species on the probability that the queens survived and produced a brood (Estimate = 0.17, SE = 1.13, z = 0.15, p = 0.88), nor on the number of offspring produced by the queens (Estimate = -0.17, SE = 1.64, z = -0.10, p = 0.92). In addition, viable hybrid workers, winged queens and males were repeatedly sampled in field colonies (Table S2).

Dyadic encounters

In encounters with non-nestmates, workers showed species recognition abilities (Figure 2). Workers aggression varied according to the species of their opponents (focal species *F. cinerea*: χ^2 = 24.35, p < 0.0001; Hybrid: χ^2 = 9.2, p = 0.01; *F. selysi*: χ^2 = 9.95, p = 0.007; Figure 2). Overall, *F. cinerea* workers showed little aggression toward conspecific workers and hybrid workers, but were aggressive toward *F. selysi* workers (post hoc analyses: *F. cinerea*-*F. cinerea* vs *F. cinerea*-Hybrid: Estimate = 0.07, SE = 0.42, z = 0.15, p = 0.88; *F. cinerea*-*F. selysi* vs. *F. cinerea*-*F. cinerea*: Estimate = 1.47, SE = 0.36, z = 4.14, p < 0.001; *F. cinerea*-*F. selysi* vs. *F. cinerea*-Hybrid: Estimate = 1.54, SE = 0.39, z = 3.98, p < 0.001). By contrast, *F. selysi* workers were aggressive toward both *F. cinerea* and hybrid workers, but less aggressive toward conspecific workers (post hoc analyses: *F. selysi*-*F. cinerea* vs *F. selysi*-Hybrid: Estimate = 0.12, SE = 0.24, z = 0.48, p = 0.63; *F. selysi*-*F. selysi* vs. *F. selysi*-*F. cinerea*: Estimate = -0.73, SE = 0.24, z = -3.05, p = 0.009; *F. selysi*-*F. selysi* vs. *F. selysi*-Hybrid: Estimate = -0.61, SE = 0.27, z = -2.32, p = 0.034). Reciprocally, hybrid workers were more aggressive toward *F. selysi* workers than toward *F. cinerea* workers, but were as aggressive toward other hybrid workers as toward workers of the two parent species (post hoc analyses: Hybrid-*F. cinerea* vs Hybrid-

Hybrid: Estimate = -0.32, SE = 0.42, z = -0.77, p = 0.44; Hybrid-*F. selysi* vs. Hybrid-*F. cinerea*: Estimate = 1.11, SE = 0.38, z = 2.92, p = 0.014; Hybrid-*F. selysi* vs. Hybrid-Hybrid: Estimate = 0.79, SE = 0.40, z = 1.97, p = 0.079). The level of aggression exhibited by each worker was positively correlated to the chemical distance between the hydrocarbon profile of its colony and that of its opponent (Spearman correlation test between aggression index and Bray-Curtis distance of chemical profiles, using one randomly-chosen observation per dyad: rho = 0.30; n = 140, $p < 0.0002$).

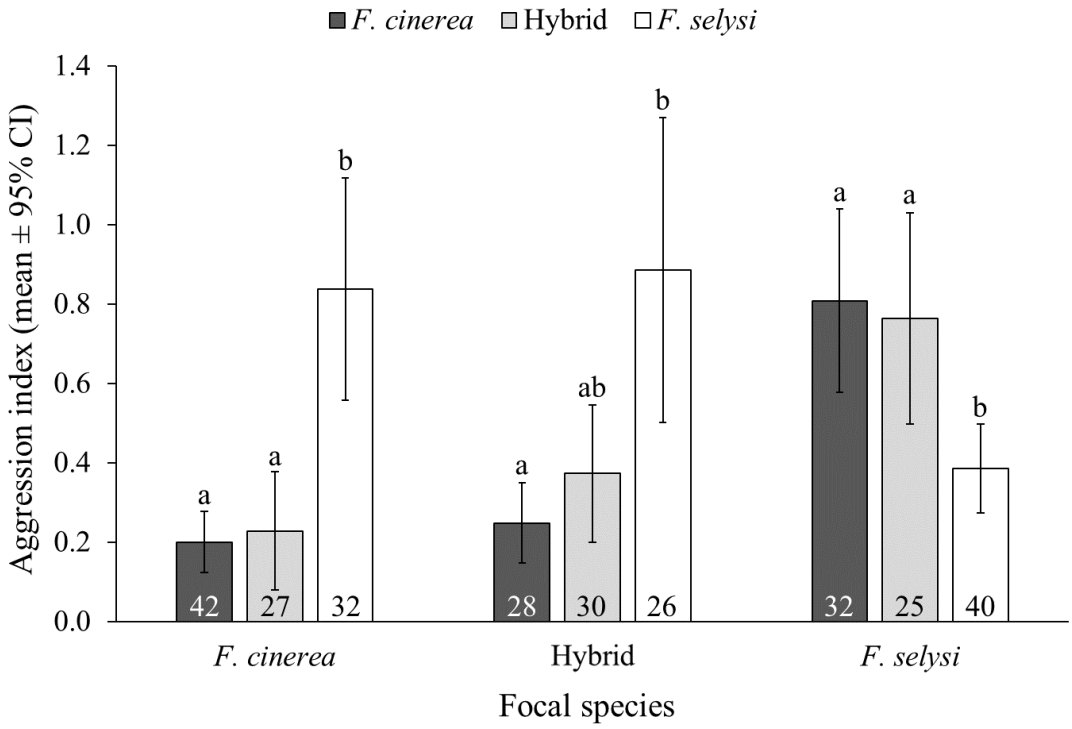

Figure 2. Aggression index of focal *F. cinerea*, Hybrid and *F. selysi* workers (x axis) according to the species of the opponent workers (*F. cinerea*: black bars; hybrids: light grey bars; *F. selysi*: white bars). Sample size is indicated within bars. Different letters indicate statistically significant differences ($p < 0.05$) after FDR correction for multiple comparisons.

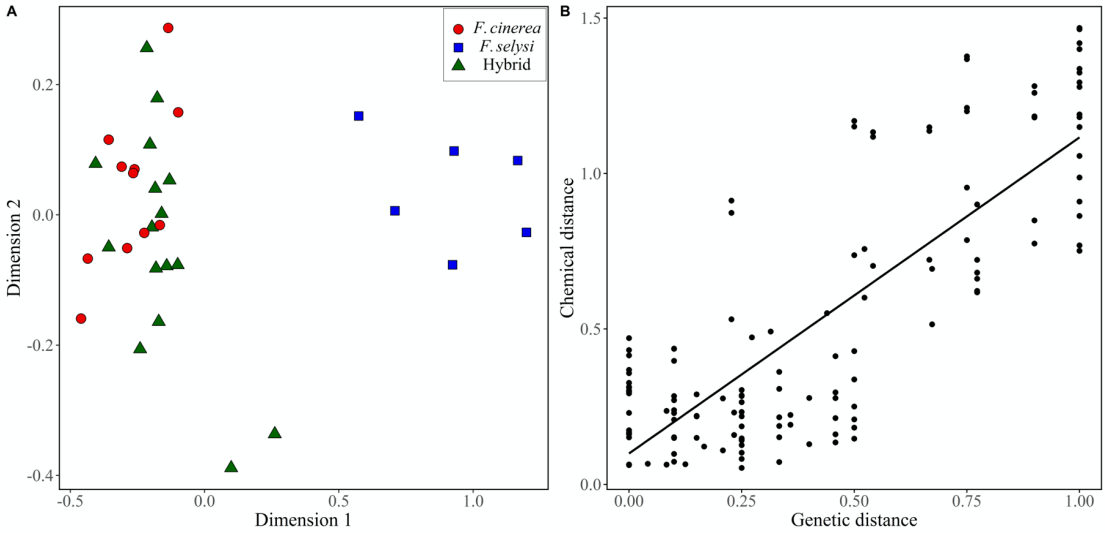

Figure 3. Proximity between cuticular hydrocarbon (CHCs) profiles of *F. cinerea*, *F. selysi* and hybrid workers. Panel (A) shows multidimensional scaling (MDS) plot of the Bray-Curtis distance between CHCs (*F. cinerea*; red circles, n = 11; *F. selysi*: blue squares, n = 6; and hybrid: green triangles, n = 16). *F. selysi* workers are perfectly discriminated from the other species and hybrids along the first dimensional axis, whereas *F. cinerea* and hybrid workers largely overlap. Panel (B) shows the positive correlation between the genetic distance and the chemical distance between colonies. Genetic distance corresponds to the difference between averaged hybrid indexes of three workers per colony and chemical distance corresponds to the Bray-Curtis distance between the averaged CHCs of two pools of three workers per colony (except one *F. cinerea* colony that had only one pool). Each dot represents one colony pair.

Cuticular hydrocarbons profiles

The CHC profiles of workers differed between species (Figure 3). In line with aggression results from dyadic encounters, *F. selysi* workers had chemical profiles that differed from those of both *F. cinerea* and hybrid workers (PERMANOVA on Bray-Curtis distances; $F_{2, 16} = 12.83$, $p < 0.001$; Figure 3a; post-hoc comparisons: both $p < 0.02$), whereas *F. cinerea* and hybrid workers had similar chemical profiles (post-hoc comparison: $p = 0.19$). The genetic distance between colonies was positively correlated to their chemical distance (Spearman correlation test between hybrid index difference and Bray-Curtis distance between chemical profiles, using one

observation per pair of colonies: rho = 0.69; n = 136, $p < 0.0001$, Figure 3b). This positive correlation was also present when considering hybrid colonies only (rho = 0.58; n = 28, $p < 0.0011$; Figure S3).

Discussion

A mosaic hybrid zone characterized by a low level of asymmetric hybridization between two ant species provides a rare opportunity to study the evolutionary mechanisms maintaining the genetic integrity of hybridizing species (Purcell *et al.* 2016; Irwin 2020). We studied potential pre- and post-mating barriers to hybridization between these two species, *F. selysi* and *F. cinerea*. Both species produced queens or males simultaneously, which suggests that temporal isolation does not prevent interspecific mating in the field. In mate choice experiments, mating was strongly assortative. Queens and males of each species preferentially mated with conspecifics, which likely plays a role in keeping hybridization low and preventing genetic admixture. We found no evidence for genetic incompatibility reducing the fertility of queens mated to heterospecific males: their hybrid offspring workers were as numerous as pure-bred offspring workers. Workers discriminated species, and their level of aggression correlated with interspecific differences in cuticular hydrocarbon profiles. *F. cinerea* and hybrid workers had similar chemical profiles and showed little mutual aggression, which is consistent with the documented asymmetric hybridization pattern (Purcell *et al.* 2016). We propose that the well-developed recognition system of workers facilitated the establishment of assortative mate preference.

In many insects, temporal isolation is a key barrier to interspecific mating (Harrison & Arnold 1982; Harrison 1983; Hölldobler & Wilson 1990; Ramsey *et al.* 2003). The monitoring of queen and male production by field colonies showed that *F. selysi* and *F. cinerea* reproductive individuals are produced in synchrony. The fact that the nuptial flights of both species occur

during the same period suggests that temporal isolation does not contribute to reproductive isolation between these species.

With mate choice experiments, we found that queens and males of *F. selysi* and *F. cinerea* preferentially mated with conspecifics. On average, 84% of all matings were intraspecific, while there were equal opportunities for interspecific mating. Strong preference for conspecifics is in accordance with the relative rarity of hybrids in the wild (Purcell *et al.* 2016). In ants, females typically attract males using volatile sex pheromones (see Walter *et al.* 1993). At closer range, non-volatile chemical cues, in particular cuticular hydrocarbons, may serve as mate recognition signals (reviewed in Howard & Blomquist 2005; Weiss *et al.* 2015). We found that *F. selysi* and *F. cinerea* workers have very distinct CHC profiles, confirming preliminary evidence from a previous study (Purcell *et al.* 2016). These CHCs convey information about species membership and may be the recognition cues underlying assortative mate choice by *F. selysi* and *F. cinerea*.

Theory and empirical data suggest that assortative mate choice co-evolves with genetic incompatibilities, in a reinforcing feedback loop (Liou & Price 1994; Servedio & Noor 2003; Albert & Schluter 2004; Dieckmann *et al.* 2012; Shizuka & Hudson 2020). In short, hybridization costs select for intra-specific mate choice, which in turn limits gene flow and increases genetic differentiation between sister species, further enlarging the costs of hybridization and facilitating species recognition (Coyne & Orr 2004). We did not detect any significant hybridization cost when comparing the fertility of interspecific crosses to that of intraspecific crosses. Mating with the other species did not decrease the queens' likelihood of producing a brood, nor the number of adult workers produced. In the few experimental studies of interspecific mating that have been conducted in ants, the outcomes were highly variable, ranging from complete lethality to fully viable hybrids (Feldhaar *et al.* 2008). Deleterious effects of hybridization may appear with backcrosses (Schwander *et al.* 2008), or when

producing queens and males rather than workers (Kulmuni & Pamilo 2014). Moreover, hybridization costs are likely to be higher in other social and ecological conditions, for example during independent colony founding by queens in harsh field conditions. Yet, genetic analyses of individuals collected in the field revealed that F1 and backcrossed hybrid workers, males and winged-females are viable in nature (Purcell *et al.* 2016; this study). Together, these data suggest that assortative mate preference evolved in absence of large hybridization costs.

If hybridization costs are moderate, why do queens and males mate preferentially with conspecifics? We suggest that the advanced recognition system of ants could have facilitated the establishment of assortative mate preference. As a result of kin selection, ants have well-developed CHC-based nestmate recognition abilities that typically manifest in being intolerant toward individuals with dissimilar CHC profiles (Guerrieri & D'Ettorre 2008). In dyadic encounters, *F. selysi* and *F. cinerea* workers were generally more tolerant toward conspecific workers, which have similar CHC profiles, but were very aggressive toward heterospecific workers, which have dissimilar CHC profiles. The hydrocarbon cues tend to diverge between species as a result of drift, local adaptation or sexual selection, and this has been shown to affect mate choice and increase reproductive isolation in various insect species (Blows & Allan 1998; Schwander *et al.* 2013; Maroja *et al.* 2014). The effective recognition system of ants, coupled with increasing divergence in recognition cues, could thus trigger queens and males to mate with conspecifics and reject heterospecifics, even in the absence of hybridization costs.

The behavioral interactions between hybrid workers and workers of each parental species were asymmetric. Hybrid and *F. cinerea* workers usually interacted peacefully, but responded aggressively to *F. selysi* workers. This behavioral pattern matches their cuticular hydrocarbon profiles, with hybrid workers being more similar to *F. cinerea* than to *F. selysi*. It is also in line with their genetic background, as the large majority of hybrids were genetically closer to *F. cinerea* than to *F. selysi*, in accordance with previous observations (Purcell *et al.* 2016). The

factors causing this skewed distribution are yet unknown. The *F. cinerea* CHC profile might be dominant in F1 hybrids, which would favor subsequent backcrosses with *F. cinerea*. The high correlation between the CHC profile of individuals and their index of hybridization provides no support to this hypothesis and rather suggests that the CHCs are co-dominant. Whatever the mechanism and causal relationships, the chemical and genetic proximity between hybrids and *F. cinerea* is associated with an asymmetric response of hybrid workers toward their parental species. This likely impacts the dynamics of the hybrid zone by reinforcing the introgression of *F. selysi* alleles into *F. cinerea*.

In conclusion, we uncovered strong, though incomplete, assortative mate choice in two hybridizing ant species, *F. selysi* and *F. cinerea*. The marked preference to mate with conspecifics helps explain the low frequency of hybrids in nature (Purcell *et al.* 2016). The absence of strong genetic incompatibilities suggests that assortative mate choice evolved in the absence of reinforcement (Servedio & Noor 2003). We propose that the CHC-based nestmate recognition mechanism used by workers facilitates the establishment of assortative mate preference in the social insects, even in the absence of large hybridization costs. Asymmetries in cuticular hydrocarbon profiles and aggression between hybrid, *F. cinerea* and *F. selysi* workers confirmed a similar asymmetry in hybridization, skewed toward *F. cinerea*. These two ant species appear to have effective recognition systems that affect both worker behavior and mate choice, with consequences at the group, population and species-levels.

Acknowledgments

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Supplementary materials

Table S1. Number of queens included in the genetic incompatibility experiment

	F. selysi queens		*F. cinerea* queens	
Mated with	*F. selysi*	*F. cinerea*	*F. selysi*	*F. cinerea*
Total	134	15	25	24
Alive after 6 weeks	126	15	21	24
Producing brood	68	8	12	11

Table S2. Field monitoring of hybrid colonies and production of hybrid winged queens and males

Year	Population	Number of field visits	Number of colonies visited	Number of hybrid colonies producing winged queens or males	Number of hybrid winged queens	Number of hybrid males
2014	Riddes	10	41	4	13	19
2014	Saillon	10	40	1	4	0
2015	Branson	13	27	8	6	9
2018	Branson	6	22	9	23	16

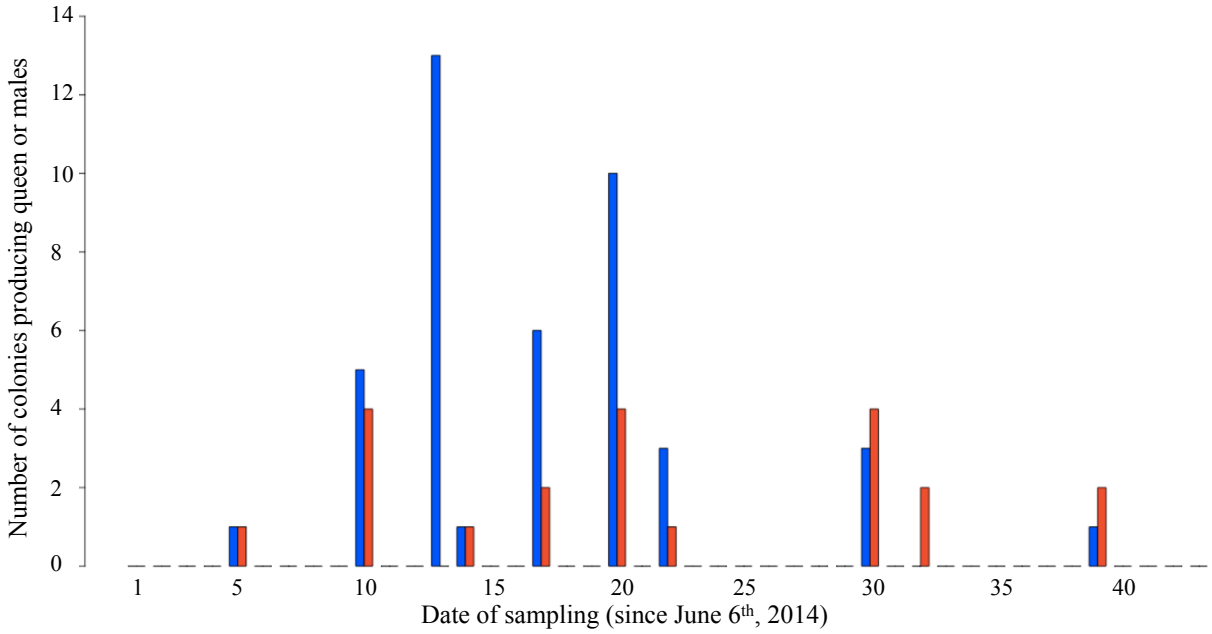

Figure S1. Timing of production of queens or males by *F. selysi* (blue) and *F. cinerea* (red) colonies. The number of colonies producing queens or males is plotted against the date of sampling (number of days since June 6th, 2014).

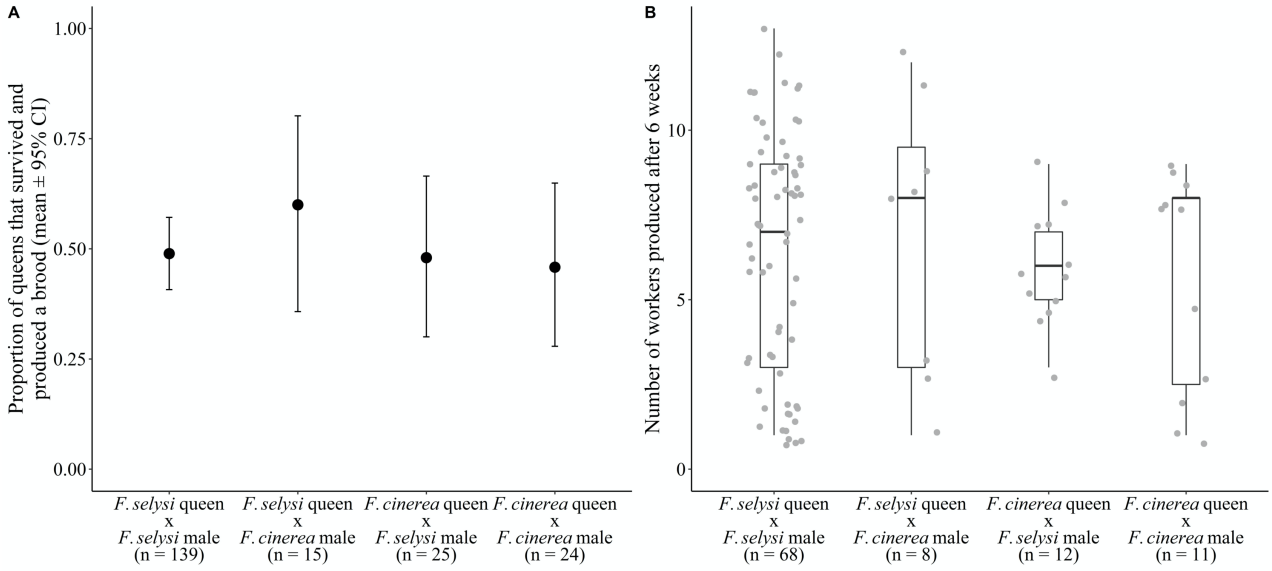

Figure S2. Reproductive success of the queens according to the species of the queen and the species of the male with whom she mated. Plot (A) shows the success of the queens at surviving and producing a brood after 6 weeks. Data are shown as mean ± SE. Plot (B) shows the number of workers produced after 6 weeks by the queens that survived and produced a brood. Horizontal lines represent the median and the boxes represent the 1st and 3rd quartiles.

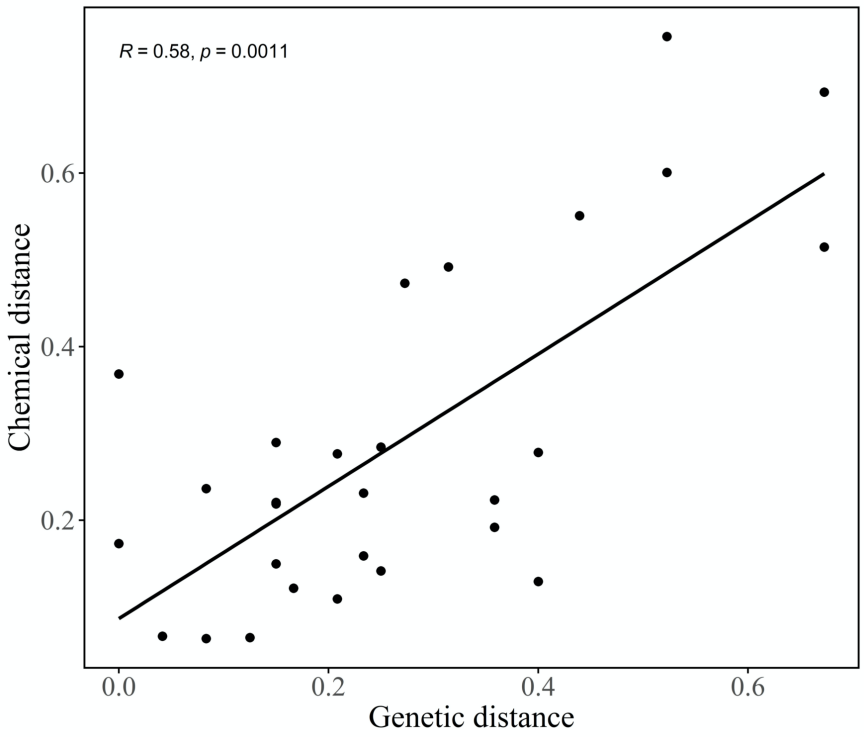

Figure S3. Correlation between the genetic distance and the chemical distance between hybrid colonies. Genetic distance corresponds to the difference between averaged hybrid indexes of three workers per colony and chemical distance corresponds to the Bray-Curtis distance between the averaged cuticular hydrocarbon profiles of two pools of three workers per colony. Each dot represents one colony pair.

Chapter 3

Fine-scale habitat heterogeneity favours the coexistence of supergene-controlled social forms in *Formica selysi*

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Authors' contributions

SZ, AF, MK and MC planned and designed the study. MK, AC, AF and SZ performed field sampling and laboratory work. MK did ant species determination. SZ and AF analyzed the data. AF, SZ and MC wrote the manuscript. All authors read and approved the manuscript.

Abstract

Background: Social insects vary widely in social organization, yet the genetical and ecological factors influencing this variation remain poorly known. In particular, whether spatially varying selection influences the maintenance of social polymorphisms in ants has been rarely investigated. To fill this gap, we examined whether fine-scale habitat heterogeneity contributes to the co-existence of alternative forms of social organization within populations. Single-queen colonies (monogyne social form) are generally associated with better colonization abilities, whereas multiple-queen colonies (polygyne social form) are predicted to be better competitors and monopolize saturated habitats. We hypothesize that each social form colonizes and thrives in distinct local habitats, as a result of their alternative dispersal and colony founding strategies. Here, we test this hypothesis in the Alpine silver ant, in which a supergene controls polymorphic social organization.

Results: Monogyne and polygyne colonies predominate in distinct habitats of the same population. The analysis of 59 sampling plots distributed across six habitats revealed that single-queen colonies mostly occupy unconnected habitats that were most likely reached by flight. This includes young habitats isolated by water and old habitats isolated by vegetation. In contrast, multiple-queen colonies were abundant in young, continuous and saturated habitats. Hence, alternative social forms colonize and monopolize distinct niches at a very local scale.

Conclusions: Alternative social forms colonized and monopolized different local habitats, in accordance with differences in colonization and competition abilities. The monogyne social form displays a colonizer phenotype, by efficiently occupying empty habitats, while the polygyne social form exhibits a competitor phenotype, thriving in saturated habitats. The combination of the two phenotypes, coupled with fine-scale habitat heterogeneity, may allow

the coexistence of alternative social forms within populations. Overall, these results suggest that spatially varying selection may be one of the mechanisms contributing to the maintenance of genetic polymorphisms in social organization.

Introduction

Genetic polymorphisms controlling phenotypic variation within species are widespread in nature, yet in many cases the mechanisms balancing these polymorphisms are unclear and remain debated (Llaurens *et al.* 2017; Faria *et al.* 2019). Selection varying in space has long been claimed to be an important force maintaining polymorphisms in natural populations (Hedrick 2006). Spatially varying selection occurs when the habitat is heterogenous and alternative alleles have different fitness in distinct habitats (Levene 1953; Bulmer 1972). Examples of polymorphisms maintained by spatially varying selection come from a large diversity of taxa. They include resistance to viruses in bacteria (Vos *et al.* 2009), shell colour in molluscs (Cain & Sheppard 1950), dispersal abilities in crustaceans (Véliz *et al.* 2006) and in insects (Wheat *et al.* 2011), colour mimetism (Joron *et al.* 1999) and resource preference in insects (Chakraborty & Fry 2016).

Social insects vary widely in social organization. Many species have a monogyne social organization, with a single breeding queen per colony. Species with a polygyne social organization, where multiple queens share reproduction in each colony, are also common, particularly in ants (Bourke & Franks 1995). And some species are socially polymorphic, exhibiting both single-queen and multiple-queen colonies, in separate or even within populations (Bourke & Franks 1995). Recent studies in ants uncovered that variation in colony social organization within species is controlled by supergenes in at least three independent lineages (Wang *et al.* 2013; Purcell *et al.* 2014b; Braims 2015; Brelsford *et al.* 2020; Yan *et al.* 2020). This strong genetic basis raises novel questions on the mechanisms maintaining social polymorphisms in time and space. In particular, it is unclear to what extent ecological factors play a role in the maintenance of social polymorphisms.

Spatially varying selection is expected if the monogyne and polygyne social forms differ in their capacity to disperse, reach and settle in different habitats. Striking differences in dispersal and colony founding occur between single-queen and multiple-queen ant species (Hölldobler & Wilson 1977; Rosengren *et al.* 1993; Ross 2001). In general, queens of monogyne species disperse on the wing and establish new colonies independently, while queens of polygyne species frequently seek adoption in their natal nests or found new multiple-queen colonies by dispersing on foot with workers (colony budding; Seppä & Pamilo 1995a; Sundström *et al.* 2005a; Cronin *et al.* 2013). Similar differences in dispersal and colony founding strategies have been documented between queens of alternative social forms within polymorphic species (Ross & Shoemaker 1997; Seppä *et al.* 2004; Gyllenstrand *et al.* 2005; Wolf & Seppa 2016; Avril *et al.* 2019a). These differences in dispersal and colony founding may lead to alternative social forms occupying distinct habitats within populations.

Single-queen colonies (monogyne social form) are predicted to have better colonization abilities, whereas multiple-queen colonies (polygyne social form) are predicted to be better competitors and monopolize saturated habitats. A general view is that ant colonies recruit additional queens in environments with high cost of independent colony founding, for example in saturated habitats with high density of ant colonies or with continuous vegetation (i.e. the habitat saturation hypothesis; Nonacs 1988a; Kokko & Lundberg 2001). According to this hypothesis, queens of multiple-queen colonies, which can join existing colonies, will be better at colonizing saturated habitats (competitor phenotype). Polygyne colonies may also have other competitive advantages, due to larger colony size , longer colony lifespan and greater genetic diversity leading to better division of labour or disease resistance (Rosset & Chapuisat 2007; Hughes *et al.* 2008; Reber *et al.* 2008). In contrast, single-queen colonies may preferentially occupy young habitats with available space to found new colonies independently (Herbers 1986; Rosengren *et al.* 1993; Bourke & Heinze 1994). In addition, due to their higher dispersal

abilities, queens of the monogyne social form may be better at colonizing patchy habitats that need to be reached by flight (colonizer phenotype), whereas polygyne colonies may thrive in continuous and connected habitats. Heterogenous mosaic landscapes, comprising a juxtaposition of empty and saturated, connected and discontinuous habitat patches, may thus favour the coexistence of genetically determined social forms varying in social organization, dispersal and mode of colony founding.

Whether the trade-off between competition and colonization favours the coexistence of alternative social forms within species remains untested. Colonization-competition trade-offs are often discussed in the framework of species coexistence, where a species occupies the "colonizer niche" by efficiently colonizing empty habitats, and the other the "competitive niche" by outcompeting the first species locally (Levins & Culver 1971; Hastings 1980; Tilman 1994; Pacala & Rees 1998). Two studies, one experimental and the other theoretical, suggest that such trade-offs play a role in the coexistence of ant species with alternative modes of dispersal and colony founding strategies (Stanton *et al.* 2002; Cronin *et al.* 2016). Here, we investigate if competition and colonization in heterogeneous habitats plays a role in the coexistence of social forms within one ant species.

The Alpine silver ant, *Formica selysi*, has a polymorphic social organization, with both monogyne and polygyne colonies (Chapuisat *et al.* 2004). Most well-sampled populations have both social forms (Chapuisat *et al.* 2004; Purcell *et al.* 2015), suggesting that the polymorphism is present at a fine geographical scale. This social polymorphism is controlled by a supergene (Purcell *et al.* 2014b; Avril *et al.* 2019a). The supergene is ancient, as it underlies the polymorphic social organization of four other *Formica* species, separated by 20 - 40 MY of independent evolution (Brelsford *et al.* 2020). In these species, single-queen and multiple-

queen colonies differ in a suite of traits, including dispersal and colony founding strategies (Rosengren *et al.* 1993; Bourke & Franks 1995; Sundström 1995).

Multiple lines of evidence suggest that *F. selysi* queens originating from monogyne colonies disperse on the wing and found colonies independently, while queens originating from polygyne colonies favour their additional option of staying in their natal colony. Monogyne colonies produce the vast majority of the queens that disperse on the wing to join mating aggregations (Rosset & Chapuisat 2006, 2007). Queens originating from polygyne colonies also fly in the field (Rosset & Chapuisat 2006) and can found colonies independently in protected laboratory conditions (Reber *et al.* 2010), although they are less successful at independent colony founding than queens from monogyne colonies, which have a larger body size (Rosset & Chapuisat 2007; De Gasperin *et al.* 2020). Nestmate queens from polygyne colonies are significantly related on average, which indicates that at least part of them stay within or close to their natal colony (Avril *et al.* 2019a). In contrast, monogyne colonies keep only one reproductive queen for their entire lifespan and mature polygyne colonies do not accept queens issued from monogyne colonies (Chapuisat *et al.* 2004; Purcell *et al.* 2014b; Avril *et al.* 2019a). Hence, unlike polygyne queens, monogyne queens do not have the possibility to join an existing nest and are obligate dispersers.

F. selysi is a pioneer species that lives in heterogeneous floodplains along rivers in the Alpine region and nests in bare sandy soils (Lude *et al.* 1999; Seifert 2003). Flood plains are dynamic and rapidly evolving areas. Ecological succession after floods creates a gradient of young to mature ecosystems representing a mosaic of habitats within small geographic areas (Ward *et al.* 1999). Major floods erode soil and eliminate ant nests, creating empty patches available for re-colonization, with varying connectivity due to water bodies. Ant and other arthropod communities typically vary among habitat patches of this mosaic landscape (Ballinger *et al.*

2007; Tagwireyi *et al.* 2015). Alternative forms of ant social organization may differ in their distribution across empty and saturated habitat types found in mosaic floodplains.

Here, we investigate if fine-scale habitat heterogeneity correlates with the distribution of supergene-mediated social forms in *F. selysi*. We search for ecological variables predicting the frequency of single-queen and multiple-queen colonies across patches of habitat, with a focus on the role of habitat age, vegetation (ecological succession) and connectivity (islands vs mainland). Due to the differences in dispersal and life-history between social forms, we expect the monogyne form to monopolize young or unconnected habitats, such as islands or recently flooded areas. In contrast, because of budding and additional queen recruitment, we predict the polygyne social form to monopolize patches of old, saturated and connected habitats.

Methods

Study site and habitat characteristics

Our study site is a floodplain along the Rhône river, within the Pfyn-Finges Nature Park, in the Valais region, Switzerland (46.311° N, 7.605° E). It comprises a large population of *F. selysi* within a 2 by 1 km area of mosaic habitat (Chapuisat *et al.* 2004; Purcell & Chapuisat 2013). Recurrent floods have created a gradient of habitats differing in age and ecological succession stages, from the riverbed to mainland approximately 1 km away from shore. Islands and frequently flooded riverbanks are characterized by a mix of bare sand and gravels, with limited vegetation. Mainland areas that are seldom or never flooded are increasingly covered by vegetation, from steppe to pine forest. Nests of *F. selysi* are typically found in bare sandy soil, usually around or under rocks (Lude *et al.* 1999).

We characterized spatial heterogeneity according to habitat age (i.e. time from the last flood), connectivity and vegetation type (Table 1). We determined the date and extent of past floods by looking at orthophotos and high-resolution satellite images from 1980 to 2016 (Swisstopo, aerial photos of 1980, 1998 and 2000; and Google Earth satellite images of 2009, 2013 and 2016). We then classified the area in six contrasted habitat categories (Table 1). Due to the selective sampling strategy, some riverine habitats covered by dense vegetation were not included. Islands are flooded yearly and remain permanently isolated by running water. As they remain under water for several days to weeks, it seems unlikely that ant colonies would survive floods, which is also suggested by the small size of colonies found on islands and riverbeds (pers. obs.). Hence, we assume that island habitat can only be colonized by flying. The riverbed is also flooded yearly, but is otherwise connected to the mainland, and can thus also be reached by foot. Parts of the mainland had been severely flooded in 2000 and in 2008, 16 and eight years before our sampling, respectively. The rest of the mainland, which had not been affected by severe floods over the last 36 years, was divided between steppe dominated by herbaceous vegetation and forest dominated by pine trees.

Sampling strategy

To investigate if colony social organization varies across habitats, we set up 59 sampling plots of 10 x 10 m, distributed across the six habitat categories (Table 1). The position of each plot within each habitat category was determined randomly using the random points function implemented in the computer program QGIS (version 2.14, QGIS 2016). The minimum distance between plots was 25 m. To further characterize the habitat, we measured within each plot the proportion of surface covered by sand, gravel, rock, moss, grass, bushes (less than 50 cm high) or trees. The plots clustered according to the six habitat categories in a Principal Component Analysis (PCA) based on their surface cover, which confirmed that these

categories differed in substrate and vegetation cover (Figure S2). The entire dataset is archived on Dryad, DOI: https://doi.org/10.5061/dryad.sj3tx963p.

Colony sampling took place in spring (April-May) and autumn (October), 2016. We located *F. selysi* colonies by following a systematic search procedure based on baiting. Within each plot, we placed 81 baits of tuna and honey on the nodes of a one meter grid square. We waited up to one hour to allow workers of nearby colonies to visit the baits. We then followed the ants back to their nests, and marked all colonies located within the 10 x 10 m plots. Colonies were considered distinct if their entrances were separated by at least one meter (Avril *et al.* 2019a). As ant activity depends on weather conditions, baiting was performed only on dry days and when the temperature was above 10 °C.

The social organization of each colony was determined by genotyping three workers per colony at SNPs that are diagnostic for alternative haplotypes of the social supergene (PCR-RFLP assay, developed for the same population, Finges; Purcell *et al.* 2014c). The supergene genotype is perfectly associated with the social form of mature colonies: workers from monogyne colonies have exclusively the supergene genotype Sm/Sm, while workers from polygyne colonies have one or two copies of the Sp haplotype (Purcell *et al.* 2014b; Avril *et al.* 2019a; Brelsford *et al.* 2020). For the 45 plots sampled in the spring, we assessed the presence of other ant species (Table S1). Workers from other ant species collected on baits were determined by examining their morphology under binocular magnifier and following identification keys (Seifert 2007; Blatrix *et al.* 2013).

Statistical analyses

To investigate if the frequency of social forms differed across habitat categories, we ran a binomial Generalized Linear Model (GLM) with the number of monogyne and polygyne colonies per plot as response variable and habitat category as fixed factor. We considered only

plots where *F. selysi* was present. We controlled for the sampling period (spring or autumn) by including it as a fixed factor in the model. We adjusted standard errors to account for over-dispersion (quasi-binomial function; Zuur *et al.* 2009).

To investigate the effect of the density of *F. selysi* colonies on the proportion of monogyne and polygyne colonies per plot, we ran a binomial Generalized Additive Mixed Model (GAMM), which handles nonlinear relationships between the response and predictor variables. We included the number of monogyne and polygyne colonies per plot as response variable, the number of *F. selysi* colonies as smoother fixed factor and the habitat category as random factor. We controlled for the sampling period (spring or autumn) by including it as a fixed factor in the model. We adjusted standard errors to account for over-dispersion (quasi-binomial function).

To analyze the effect of ant species diversity on the density of *F. selysi* colonies, we ran a GAMM with Poisson distribution. We included the number of *F. selysi* colonies per plot as response variable, the number of other ant species as smoother predictor, and the habitat category as random factor. All statistical analyses were performed using R 3.5.1 (R Core Team 2018). We used the package "lme4" (Bates *et al.* 2014) for GLMs and the package "mgcv" (Wood 2011) for GAMMs.

Results

F. selysi was abundant in the floodplain but had a patchy distribution across the mosaic landscape. Using a systematic search procedure, we detected 354 colonies in 59 plots of 10 x 10 m belonging to six habitat categories (Table 1). The density of *F. selysi* colonies varied greatly among plots (range: 0-22 colonies per plot) and between habitat categories (Table 1; Table S1). The species occupied all types of young habitat (islands, riverbeds, 8-year old and 16-year old flooded areas), as well as old, open steppe habitat, but was completely absent from

old, mature pine forest (Table 1). As expected, the species was socially polymorphic, with 32.6% of the colonies belonging to the monogyne social form, and 67.4% to the polygyne social form (N = 340 colonies; the social organization of 14 colonies could not be determined; Table 1).

The proportion of monogyne and polygyne colonies varied greatly between habitat categories (GLM, "habitat category": df = 42, F = 8.24, $p < 0.001$; Table 1; Figure 1). The monogyne social form was by far the most common on islands (97.7% of single-queen colonies on islands, N = 44 colonies) and in steppes (100% of single-queen colonies, N = 8 colonies). In contrast, the polygyne social form was the most common in young mainland habitats, which included riverbeds (92.3% of multiple-queen colonies, N = 26), 8-year old flooded area (85.6% multiple-queen colonies, N = 180) and 16-year old flooded area (61% of multiple-queen colonies, N = 82).

The frequency of alternative social forms was associated with different vegetation types and habitat connectivity levels (mainland versus islands). On mainland, monogyne colonies were more frequent in plots with more vegetation (covered by grass, bushes or trees), whereas polygyne colonies were more frequent in plots with mineral surface (covered by rocks, gravel or sand; Spearman correlation between proportion of mineral surface cover and proportion of polygyne colonies: S = 4420.9, rho = 0.48, $p < 0.01$). On islands, almost all colonies were monogyne. Island plots were covered by rocks, gravel or sand and were ecologically similar to riverbed plots (Figure S1). Indeed, islands and riverbeds are annually disturbed by floods and are pioneer, vegetation-poor habitats. Yet islands, which are probably only reachable by flying,

Table 1. Habitat characteristics and distribution of alternative social forms across habitat types

	Island	**Riverbed**	**Flooded area 8 yo**	**Flooded area 16 yo**	**Steppe**	**Forest**
Age since last flooding	Approx. 1 year	Approx. 1 year	8 years	16 years	> 36 years	> 36 years
Number of plots	8	11	15	10	10	5
Number of ant species	2	2	1	1	7	4
Number of colonies per plot (mean ± SD)	5.5 ± 4.1	2.36 ± 1.8	12.66 ± 6.1	8.5 ± 2.1	0.9 ± 1.7	0
Number of monogyne colonies per plot (mean ± SD)	5.38 ± 3.89	0.18 ± 0.60	1.73 ± 2.18	3.2 ± 3.94	0.8 ± 1.48	0 ± 0
Number of polygyne colonies per plot (mean ± SD)	0.13 ± 0.35	2.18 ± 1.94	10.26 ± 6.41	5 ± 4.29	0 ± 0	0 ± 0
Total number of *F. selysi* colonies (number of colonies with undetermined social form)	44 (0)	26 (0)	190 (10)	85 (3)	9 (1)	0 (0)

were almost exclusively occupied by single-queen colonies, while riverbeds, which can be reached by foot and flight, were almost exclusively occupied by multiple-queen colonies.

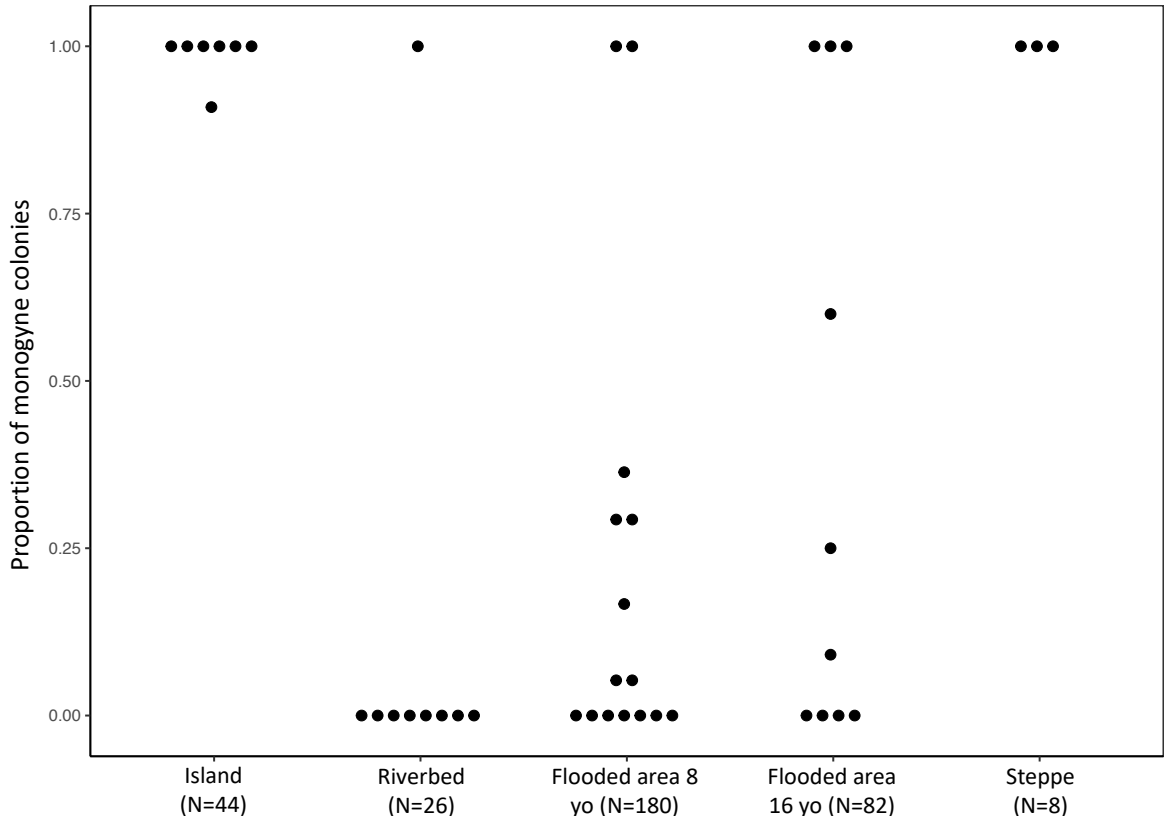

Figure 1. The frequency of social forms varies across habitat types. Monogyne colonies are more frequent on islands and in steppes, while polygyne colonies are more frequent in riverbeds and flooded areas. The proportion of single-queen colonies per plot is indicated, for each habitat category. Dots correspond to plots in which *F. selysi* colonies were sampled, and N indicates the total number of colonies sampled in each habitat category.

The frequency of social forms varied with the density of *F. selysi* colonies. Across all habitat categories, multiple-queen colonies were more frequent in plots with higher colony density (GAMM, "colony density": df = -0.97, F = 11.60, $p < 0.01$; Figure 2; Table 1). The high density of colonies was also associated with low ant species diversity (GAMM, "diversity": df = -4.19, $p = 0.03$, Table S1). In particular, *F. selysi* was the only ant species in mainland young habitats,

which are densely populated by multiple-queen colonies (8 yo and 16 yo flooded areas; Table S1; Figure 2).

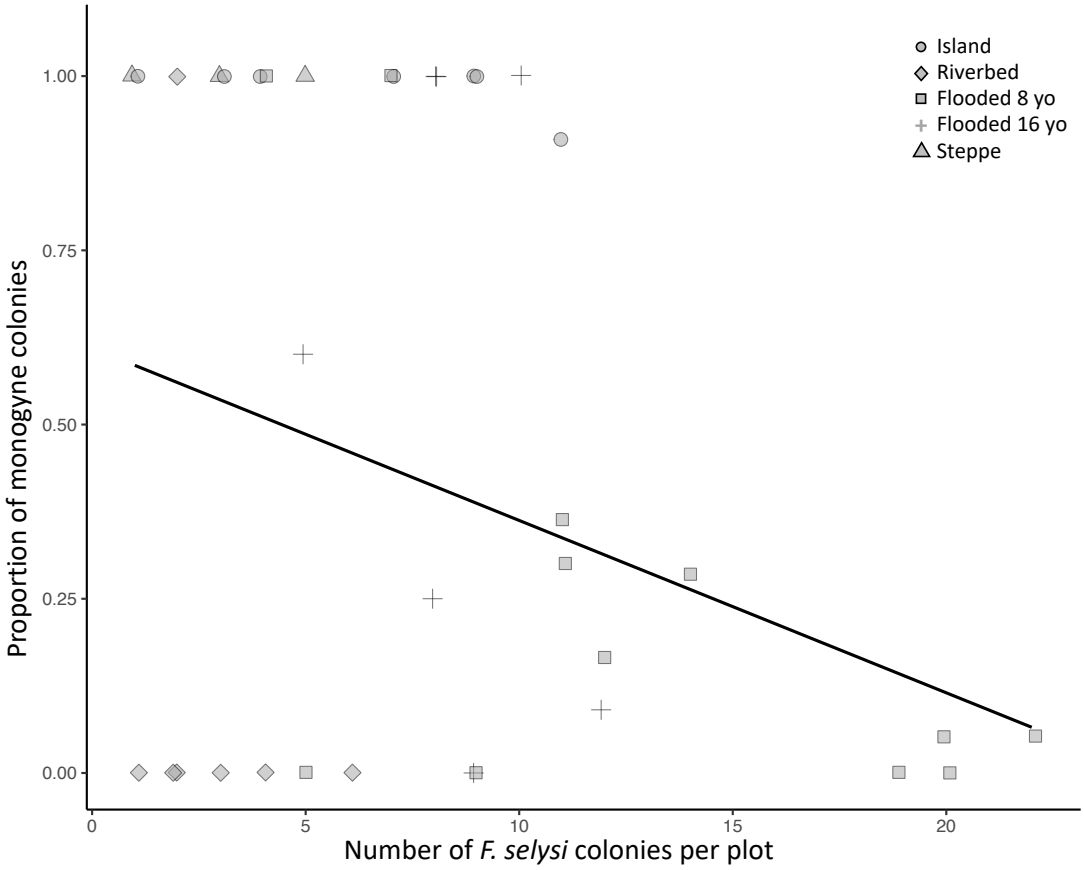

Figure 2. The frequency of social forms varies with colony density. Each point corresponds to a plot, with the symbol indicating its habitat category. The points have been jittered for better visualization. Regression curve represents a simplified GAM model, with the number of monogyne and polygyne colonies as response variable and colony density as fixed factor.

Discussion

Evolutionary forces maintaining intraspecific polymorphism, such as social organization in ants, are still poorly understood. Alternative forms of social organization are often associated with distinct dispersal and colony-founding strategies. Here, we focus on the interplay between the colonizer and competitor phenotypes of alternative social forms and their success at occupying distinct habitats. We investigated whether and how heterogeneous habitats could

favour social polymorphism at a fine geographical scale. We found that in a species with genetically determined social organization, the frequency of monogyne and polygyne social forms varied strikingly between habitat types of a mosaic floodplain. Monogyne colonies were more frequent in less populated and more isolated or disconnected habitat patches. In contrast, polygyne colonies were abundant in young, saturated and more connected habitats. This distribution is in line with different dispersal and colony founding strategies, with the monogyne social form exhibiting a colonizer phenotype and the polygyne social form presenting a competitor phenotype. Therefore, our results suggest that social forms play a distinct role in a competition-colonization trade-off, which could promote the coexistence of alternative, genetically determined social forms within populations. To our knowledge, this is the first study to report an association between habitat heterogeneity and the frequency of genetically controlled social forms in the same population.

The polygyne social form monopolized saturated habitats. This is in line with the habitat saturation hypothesis, which predicts that polygyne colonies are abundant in saturated habitats with few available nesting sites (Nonacs 1988; Kokko & Lundberg 2001). Several studies have found a positive association between the number of queens per colony and proxies of habitat saturation, such as nesting site limitation, invasion gradient and ecological succession (Herbers 1986; Bourke & Heinze 1994; Seppä *et al.* 1995; Ross *et al.* 1996; Pedersen & Boomsma 1999; Ingram 2002). Yet, these studies compared social forms in distant locations (Ross *et al.* 1996; Dalecky *et al.* 2007) or assumed that social organization was a plastic trait (e.g. Seppä *et al.* 1995a; Ingram 2002a; McGlynn 2010). Contrary to what is assumed in the habitat saturation hypothesis, monogyne colonies of *F. selysi* cannot adopt additional queens and mature polygyne colonies never accept young queens of the alternative social origin (Avril *et al.* 2019a). Thus, in our system, the abundance of multiple-queen colonies in saturated habitats is

due to the success of individuals holding the supergene variant associated with polygyny, rather than to a plastic response of colonies facing changing environmental conditions.

The monogyne social form was more successful than the polygyne one at colonizing the unconnected island habitats. Monogyne colonies represented the large majority of colonies on islands, although we also found one polygyne colony, confirming that polygyne queens have the possibility to disperse by flight. Riverbeds and islands are ecologically similar in vegetation and soil cover, and present abundant and continuous nesting sites, yet they strikingly differ in the proportion of monogyne and polygyne colonies. After severe floods, riverbeds and islands have to be recolonized by females originating from non-flooded areas. Riverbeds are connected to the mainland and may thus be efficiently colonized by workers and queens walking from nearby polygyne colonies (colony budding). By contrast, queens originating from monogyne colonies appear better at reaching and colonizing unconnected habitats.

The oldest habitat, steppes, was occupied exclusively by the monogyne social form. This does not fit the prediction of the habitat saturation hypothesis, whereby polygyne colonies should dominate old, stable habitat with low nest site availability (Herbers 1986; Bourke & Heinze 1994; Seppä *et al.* 1995; Dalecky *et al.* 2007). Steppes are old and mature habitats, with the highest diversity of ants in this floodplain. Suitable nesting sites in sandy patches are isolated amidst dense vegetation and may only be attained by flight. Females from single-queen colonies may thus have an advantage at reaching and colonizing scattered nest sites in steppes. Other traits may also contribute to a better adaptation of the monogyne social form to islands and steppes. Yet, our results suggest that habitat connectivity is a major ecological factor determining the success and distribution of alternative supergene haplotypes that affect both dispersal and social organization.

Overall, the very unequal distribution of social forms across habitat types suggests that spatially varying selection contributes to the coexistence of alternative supergene haplotypes controlling social organization, dispersal and colony founding strategies. The two social forms appear locally adapted to contrasting habitat types, as in a multi-niche selection framework for dispersal- and competition-related traits (Levene 1953). Models predict that a genetic polymorphism for dispersal can be maintained in heterogeneous environments if there is spatial variation in the carrying capacities of patches (Mathias *et al.* 2001; Massol *et al.* 2010). Dynamic floodplains show such variation, and selection in spatially heterogeneous environments can thus contribute to maintain the polymorphism within populations. We do not know whether habitat heterogeneity plays a role in the distribution of other socially polymorphic *Formica* species. The maintenance of the polymorphism over 20 - 40 MY of evolution (Brelsford *et al.* 2020) and across the species ranges (Purcell *et al.* 2015) likely requires additional mechanisms than spatially varying selection. Yet, our findings highlight the importance of taking spatial distribution and ecological features into account in the study of the evolution and maintenance of supergenes.

Conclusion

This survey links habitat characteristics to the distribution of supergene-mediated social forms in Alpine silver ants. The mosaic riverine landscape consists in habitat patches that vary in age, vegetation cover and connectivity. The frequency of monogyne and polygyne colonies varies strikingly between habitat types in the same population. Single-queen colonies occupy steppes and islands, while multiple-queen colonies strive in riverbeds and recently flooded areas. Alternative social forms appear to be adapted to colonize and monopolize distinct niches at a very local scale. Overall, these results suggest that habitat heterogeneity, coupled with strong

differences in dispersal and colony founding, help to explain the co-occurrence of alternative social forms within populations.

Aknowledgments

We thank Sagane Dind for help with laboratory work, as well as Pierre Blacher and Ornela De Gasperin for discussion on the manuscript.

Supplementary materials

Table S1. Ant species abundance across habitat categories, estimated as the proportion of plots in which a species was detected. Species presence is highlighted in bold (N = number of plots per habitat category). To test if ant species diversity varied across habitat categories, we ran a GLM with a Poisson distribution, with the number of ant species as response variable and the habitat category as predictor. The diversity of ant species differed markedly between habitat categories (GLM, "habitat category": df = -5, $p < 0.01$).

	Island (N = 3)	**Riverbed** (N = 7)	**Flooded area 8 yo** (N = 10)	**Flooded area 16 yo** (N = 10)	**Steppe** (N = 10)	**Forest** (N = 5)
Formica selysi	**0.67**	**1.0**	**1.0**	**1.0**	**0.3**	0.0
Formica cunicularia	0.0	0.0	0.0	0.0	**0.1**	0.0
Formica clara	0.0	0.0	0.0	0.0	**0.1**	0.0
Myrmica lonae	0.0	0.0	0.0	0.0	0.0	**0.2**
Manica rubida	**0.34**	**0.14**	0.0	0.0	0.0	0.0
Plagiolepis vindobonensis	0.0	0.0	0.0	0.0	**0.1**	**0.2**
Tetramorium sp	0.0	0.0	0.0	0.0	**0.1**	0.0
Tapinoma erraticum	0.0	0.0	0.0	0.0	**0.1**	**0.2**
Themnothorax parvulus	0.0	0.0	0.0	0.0	**0.1**	**1.0**

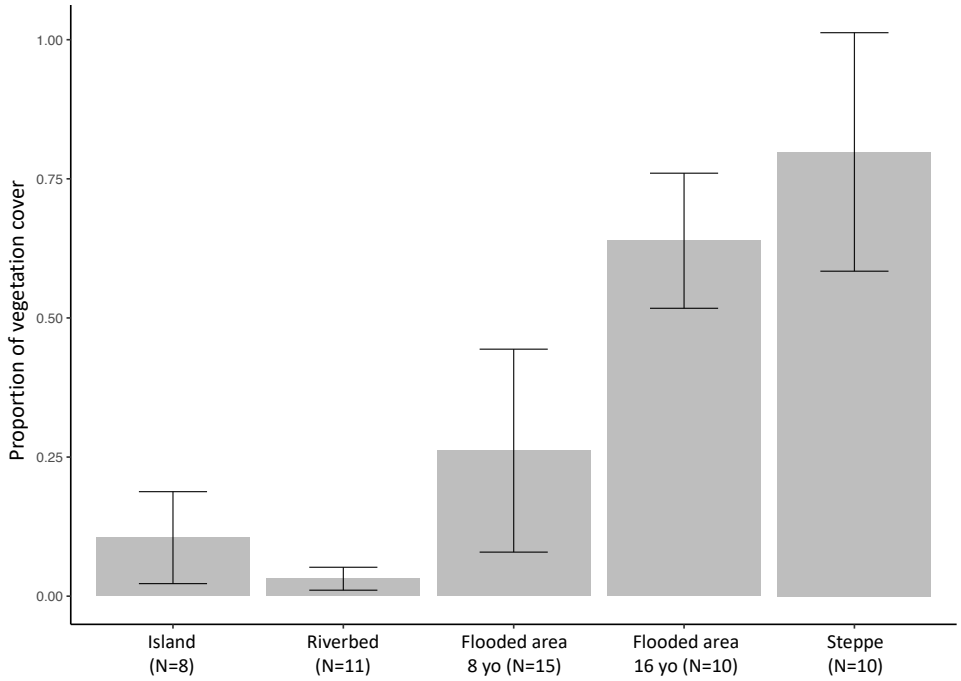

Figure S1. Vegetation cover across habitat categories, given by the mean proportion of plots' surface covered by vegetation, including moss, grass, bushes and trees (N = number of plots per category; error bars indicate standard deviation).

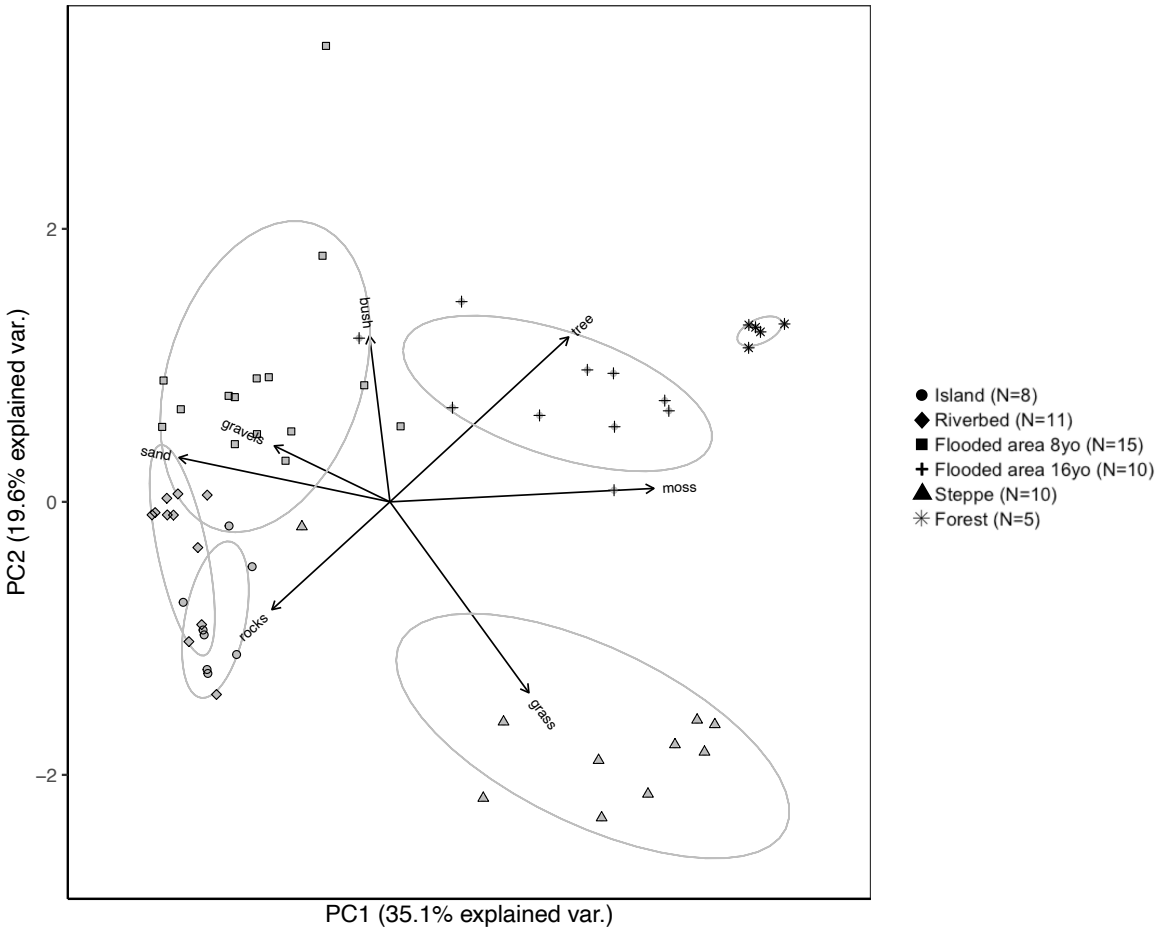

Figure S2. Surface cover across plots. Principal component analysis (PCA) of surface cover variables in the 59 sampling plots (proportion of surface covered by sand, gravel, rock, moss, grass, bushes and trees, respectively). The first component (35.1 % of the variance) mainly differentiates the substrate (vegetation versus mineral) and the second component (19.6 % of the variance) mainly differentiates low vegetation (grass) from high vegetation (bush and trees). Plots in each of the six habitat categories cluster together, which indicates that habitat categories differ in substrate and vegetation. The number of plots is indicated in parentheses.

Chapter 4

Mitogenomes and supergene are unlinked in silver ants, revealing female-mediated gene flow between alternative social forms

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Manuscript in preparation

Author's contributions

SZ, AA and MC planned and design the study, SZ and MB performed field sampling and laboratory work. SZ and PTV analyzed the data. SZ and MC wrote the manuscript with contribution from AA and PTV.

Abstract

Mating system and sex-biased dispersal have a great impact on the distribution of genetic polymorphisms. In ants, males tend to disperse, whereas females are thought to be philopatric. As theory predicts that the coevolution of dispersal and sociality would result in the formation of supergenes (i.e. clusters of tightly linked loci), the discovery of a supergene-mediated social polymorphism in the Alpine silver ant, *Formica selysi*, offers a great opportunity to investigate the role of sex-biased dispersal in the maintenance of a social polymorphism. In this species, colonies within populations can be headed by a single queen (monogyne) or by multiple queens (polygyne). Whereas male-mediated asymmetric gene flow has been shown to occur from the monogyne to the polygyne social form, polygyne colonies never produce monogyne individuals due to a maternal effect killing. So far, the presence of female-mediated gene flow remained cryptic. In this study, we take advantage of whole genome sequencing of 48 *F. selysi* workers to explore whether mitochondrial lineages are specific to social forms, and if they are inherited independently or not from the supergene. We found no or little genetic differentiation between social forms at the mitochondrial level, indicating at least occasional female-mediated gene flow. In both haplotype network and phylogenetic analyses, mitogenomes clustered according to their geographic origin, and not by social form. These results indicate that the supergene and mitochondria are unlinked in the Alpine silver ant. Female-biased gene flow likely occurs when a monogyne queen mate with a polygyne male and establish a colony that later become polygyne. Overall, this study demonstrates that comparing autosomal and sex-linked markers is a powerful tool to better understand life-cycles and mating systems.

Introduction

The mating system, and in particular sex-biased dispersal, affects gene flow and population genomic structure (Hedrick 2007). Sex-biased dispersal and its effects on the distribution of genetic polymorphism have been studied in numerous systems, including plants (Holsinger 1986; Pannell 2015), mammals, birds (Greenwood 1980; Mabry *et al.* 2013) and insects (Fortelius *et al.* 1987; Wickman & Rutowski 1999). Molecular approaches provide useful tools to uncover patterns of sex-biased dispersal. In particular, whole genome sequencing allows researchers to compare between nuclear markers (biparentally inherited) and mitochondrial markers (maternally inherited), providing an opportunity to better understand sex-biased dispersal (Shaw *et al.* 2018). For example, in mammals but also in ants, males tend to disperse while females tend to be philopatric (Matthews *et al.* 2021; Osada *et al.* 2021).

In ants, dispersal is mediated by winged males and females that will mate outside or inside their nests (i.e. colonies). Colonies are composed of females, queens and workers, that usually live in a fixed location (Hölldobler & Wilson 1990; Hakala *et al.* 2019). Females mate in a single event at the beginning of their lives and store the sperm of one or multiple males to fertilize eggs over several years. Their mating system and dispersal mode are tightly linked, as mated females will either found a colony in a new location, or stay in their mother's nest, depending on whether they mated outside or inside of their natal colony. Theory predicts that the coevolution of sociality and dispersal would result in the formation of supergenes (i.e. clusters of tightly linked loci; Mullon *et al.* 2018), offering new opportunities to understand how mating system and dispersal shape the distribution of genetic polymorphisms in eusocial insects.

The recent discovery of a supergene associated with social organization in an ant species provides an ideal opportunity to investigate how mating system and sex-biased gene flow influence the maintenance of polymorphism (Purcell *et al.* 2014b). In the Alpine silver ant,

Formica selysi, colonies can be headed by one queen (monogyne) or by multiple queen (polygyne). This variation of social organization is associated with a polymorphism at a supergene, with two haplotypes, Sm and Sp (Purcell *et al.* 2014b, 2015). In single queen (i.e. monogyne) colonies, all individuals carry exclusively the Sm haplotype (queens and workers being Sm/Sm and males Sm; Purcell et al. 2014). By contrast, in multiple queen (i.e. polygyne) colonies, all individuals carry at least one copy of the Sp haplotype (Purcell *et al.* 2014b). Specifically, queens and workers are either Sp/Sm or Sp/Sp and males are Sp. In addition, and contrary to most other organisms bearing a supergene (Schwander et al. 2014), the homozygous mutant (Sp/Sp) is viable in *F. selysi* (Purcell *et al.* 2014b; Avril *et al.* 2020). Sm males and Sm/Sm females offspring of heterozygous queens in polygyne colonies never develop due to maternal effect killing, acting as a transmission distorter and restricting gene flow between social forms (Figure 1; Avril et al. 2020). Usually, the toxin (or modification) gene and the antidote (or rescue) gene are tightly linked in the selfish genetic element. The Sp supergene haplotype of *F. selysi* displays such a maternal effect killing (Avril *et al.* 2020). It is however possible that cyto-nuclear interactions play a role in the killing, if the selfish supergene haplotype is co-transmitted with cytoplasmic elements. Examining if social forms harbor private mitochondrial lineages is a good way to examine whether the Sp haplotype and cytoplasmic elements are co-inherited. If they are not strictly associated, one can conclude (1) that there is occasional maternal gene flow between social forms, resulting in cytoplasmic introgression and (2) that cyto-nuclear incompatibilities are unlikely to play a role in the maternal effect killing.

There is little genetic differentiation between social forms of *F. selysi* at nuclear markers outside the supergene, indicating on-going gene flow (Chapuisat *et al.* 2004; Purcell & Chapuisat 2013; Purcell *et al.* 2014b). However, this gene flow is highly asymmetric, occurring primarily from the monogyne to the polygyne form, due to Sm males mating with polygyne

queens (Avril *et al.* 2019a). In the wild, nearly a quarter of queens in polygynous colonies mate with Sm males (in 22.9% of cases; Avril et al. 2019). In contrast, queens in monogyne colonies mate exclusively with Sm males (Purcell *et al.* 2014b; Avril *et al.* 2019a). Despite numerous field surveys and evidence that Sm/Sm queens and Sp males mate in nature (Fontcuberta *et al.* 2021), no mature monogyne colonies headed by a Sm/Sm queen mated to a Sp male has been observed (Purcell *et al.* 2014b; Avril *et al.* 2019a). The absence of this cross is intriguing, as Sm/Sm females and Sp males mate successfully under laboratory conditions and produce viable offspring (Reber *et al.* 2010), with the hypothesis that colonies headed by Sm/Sm queens mated with Sp males transition to polygyne colonies (Fontcuberta *et al.* 2021). This conversion would lead to a female-mediated gene flow from the monogyne to the polygyne social form. Yet, it is still to be determined whether this cross results in viable colonies in the field or whether there are barriers to mating between monogyne females and polygyne males (Figure 1).

Here, we investigated the role of mating system and potential cyto-nuclear incompatibilities in the maintenance of a supergene-mediated social polymorphism in *F. selysi*. We take advantage of the complete genomes from 48 workers issued from four populations to explore whether mitochondrial lineages are specific to social forms, and if they are co-inherited with the supergene, or occasionally segregate independently. We expect to observe co-transmission if Sm/Sm queens mated with Sp males are not able to found colonies in nature, as maternal effect killing is complete (monogyne queens are never produced in polygyne colonies; Avril *et al.* 2020). However, we expect an independent transmission of the supergene and the mitochondrial genome if they can mate in nature, found viable colonies as they do in laboratory conditions and transition to polygyne colonies (Avril *et al.* 2019b; Fontcuberta *et al.* 2021).

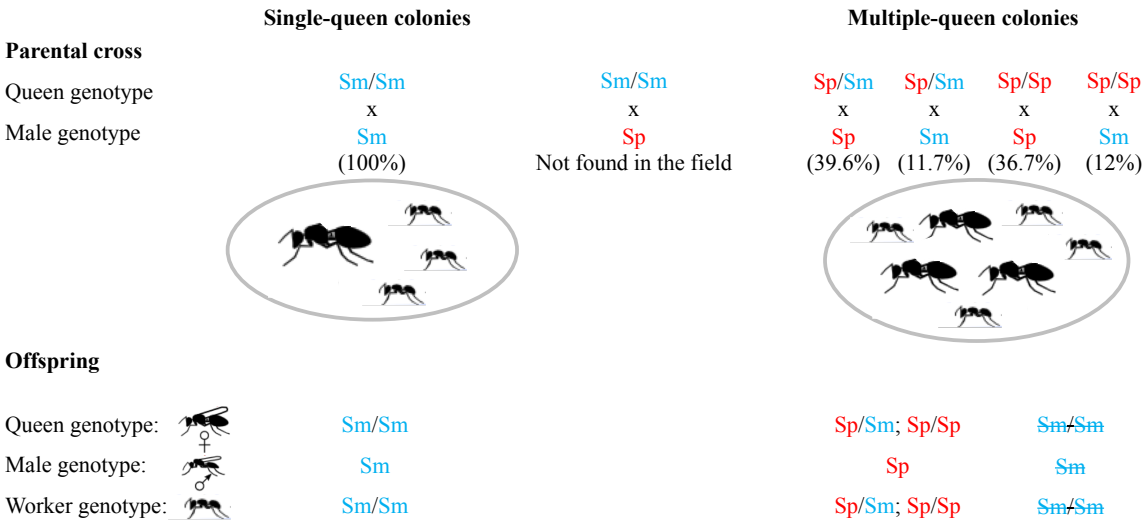


Figure 1. Genetic system underlying social organization in the Alpine silver ant, *Formica selysi* (adapted from Avril et al. 2020). The proportion of each parental cross is indicated in parenthesis, as found in the field (from 63 queens from single-queen colonies and 150 queens from multiple-queen colonies; data from Avril *et al.* 2019a). Mature colonies founded by a single Sm/Sm queen mated with Sp males are not found in the field. Sm/Sm queens and workers and Sm males are not produced by polygyne colonies.

Material and methods

Study species and samples

The Alpine silver ant, *F. selysi*, is a pioneer species that lives in floodplains along rivers in the Alpine region (Lude *et al.* 1999; Seifert 2003). To unravel the mating system and search for signs of female-mediated gene flow between social forms, we sequenced the complete genome of 48 workers, each from an independent colony, from four populations (Figure 2).

Whole-genome sequencing

We extracted the DNA using the Qiagen DNeasy kit (Qiagen, Hilden, Germany) for insects, following the manufacturer protocol. The library was prepared by the Genomic Technologies Facility at the University of Lausanne, using the NEBNext Ultra II DNA kit. The sequencing was performed on an Illumina HiSeq2500 system, with 100 bp paired-end reads. The sequencing resulted in approximatively 30 million reads per individual, corresponding to a

mean coverage of 20x before pre-processing. Published whole-genome sequencing data from two *Formica cinerea* individuals (one monogyne and one polygyne) were used as outgroup (Brelsford *et al.* 2020).

Data processing, variant calling and filtering

Individual sequencing paired-end raw reads were quality trimmed with Trimmomatic v0.36 (Bolger *et al.* 2014). Reads with a sequence length shorter than 80 bp after trimming were discarded. Filtered reads were mapped against a novel unpublished reference PacBio genome of the monogyne social form of *F. selysi* (a more contiguous version from the genome published in Brelsford *et al.* 2020). Reads were mapped and aligned using BWA-mem v0.7.17 (Li & Durbin 2009), with default parameters. Duplicates were removed using Picard Tools MarkDuplicates (Broad Institute 2018). We then processed the nuclear and mitochondrial data separately. The nuclear SNPs and indels were called with Samtools v1.10 (Li *et al.* 2009) and the module BCFtools. Further filtering was done using VCFtools v0.1.15 (Danecek *et al.* 2011). We selected only high-quality single nucleotide polymorphisms (SNP), i.e. SNPs having a genotype quality of at least 20 and a depth above 8. We then considered separately the supergene (situated on chromosome 3) and the rest of the nuclear genome. Concerning the mitochondrial data, we obtained the mitochondrial genome of each individuals using a custom script, by integrating variable sites in the reference genome (Yang *et al.* 2015) as detected after SNP calling with Samtools v0.1.15 (Danecek *et al.* 2011). To obtain the annotation and gene positions, we used MITOS (Bernt *et al.* 2013) and the annotation of the complete mitogenome from Yang *et al.* (2015).

Species validation and social form determination

To validate species determination, we used all the scaffolded nuclear SNPs, excluding the supergene, to conduct a Principal Component Analysis (PCA), and included *F. cinerea* samples as outgroup. In addition, to determine the social form of each individual, we performed a PCA on the SNPs located in the supergene. Both PCA were calculated using the plink software v1.9 (Purcell et al., 2007) and visualized in R version 4.0.2 (R Core Team 2016). In *F. selysi*, PCA on the supergene typically results in a cluster for Sm/Sm individuals, whereas Sp/Sp individuals are more spread, with Sp/Sm displaying an intermediate pattern (Brelsford et al. 2020). We further confirmed the assigned social organization with a diagnostic PCR-RFLP essays (Avril et al. 2019a and 2019b). Then, we calculated the inbreeding coefficient (F_{IS}) using the SNPs located in the supergene with VCFtools v0.1.15 (Danecek *et al.* 2011). Negative F_{IS} denotes an excess of heterozygosity and allows to separate heterozygous individuals at the supergene from homozygous ones.

Mitogenome structuration

We analyzed the relationship between the mitogenomes of *F. selysi* workers by building a haplotype network from the individual mitochondrial sequences using the "pegas" package in R (version 0.13; Paradis 2010). In addition, we assessed the phylogenetic relationship between the 48 *F. selysi* mitogenomes by building a Maximum Likelihood tree using the "phangorn" package (version 2.5.5; Schliep 2011). We used the two mitogenomes of *F. cinerea* as outgroup. We then obtained branch support values by bootstrapping 1,000 pseudo-replicates using the same package.

Finally, we compared the genetic differentiation between social forms by computing pairwise F_{ST} between the mitogenomes of workers originating from monogyne and polygyne mitogenomes within each population (Sp/Sm and Sp/Sp were considered as one group), using

the “hierfstat” package (Goudet 2005) and following the approach described in de Meeus & Goudet (2007). We obtained 95% confidence intervals by bootstrapping 1,000 times over loci.

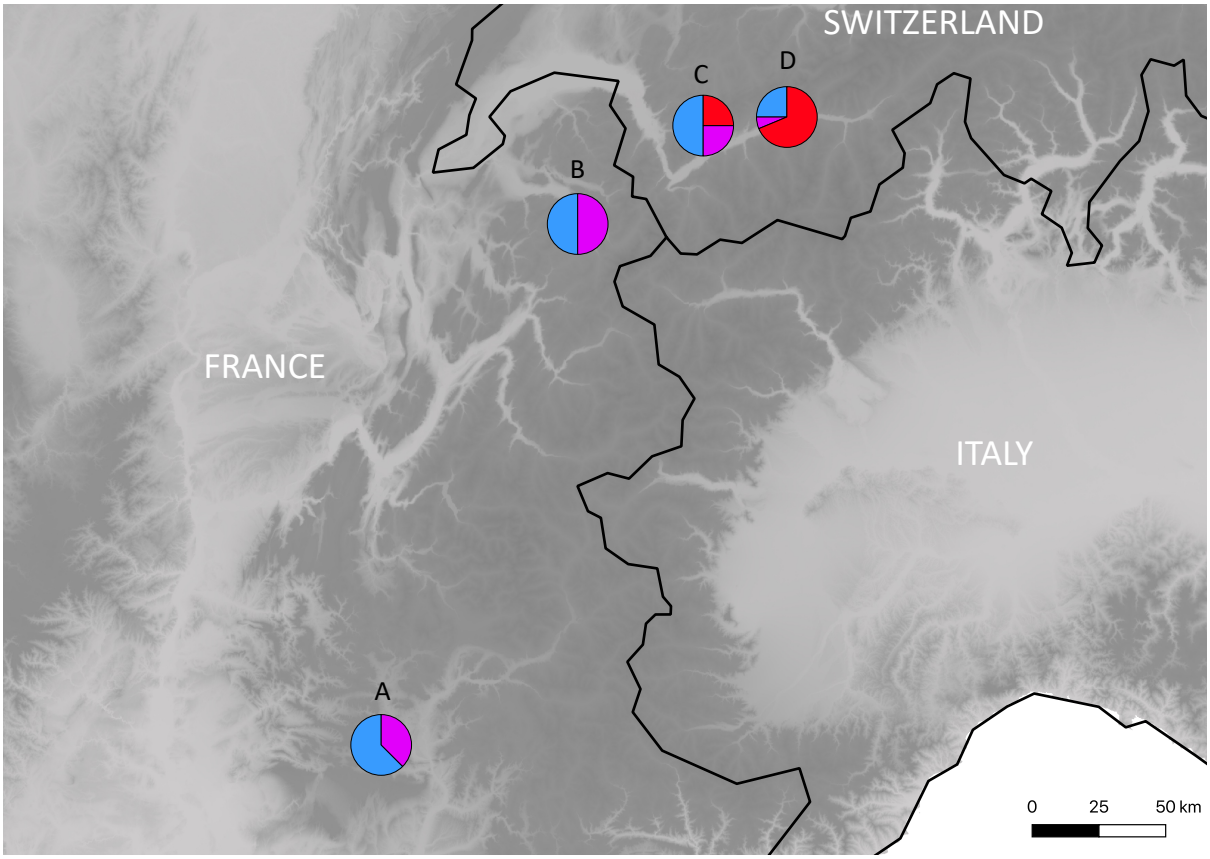


Figure 2. Sampling scheme. In total, 48 complete genomes were analyzed from four populations of *Formica selysi*, Les Bussets (A; N=8), Sallanches (B; N=8), Derborence (C; N=16) and Finges (D; N=16). Colors indicate the supergene genotypes of the sampled individuals (monogyne Sm/Sm in blue; polygyne Sp/Sm in purple and Sp/Sp in red).

Results

Across four populations, the mitogenomes from 21 Sm/Sm, 12 Sp/Sm and 15 Sp/Sp *F. selysi* workers (Figure 2; Supplementary Figure 1A and 1B) clustered according to their geographic origin regardless of social forms, both in the haplotype network and in the phylogenetic analyses (Figure 3 A and B; Supplementary Figure 2). The analysis of the complete mitogenome of *F. selysi* revealed low genetic diversity, with only 40 variables sites (SNPs) in

a genome of 16,752 bp. The SNPs were located in the genes *atp8* (2), *cob* (3), *cox1* (3), *nad1* (3), *nad2* (2), *nad4* (1), *nad4L* (2), *nad5* (2) *nad6* (1), *rrnL* (1), *rrnS* (1), *tRNA-Phe* (1) and non-coding region (16). This number of variable sites suggest a relatively low mitochondrial diversity, compared to other Hymenoptera (Table1).

The haplotype network confirmed the low level of genetic diversity in *F. selysi* mitogenomes (Figure 3). Accordingly, the phylogenetic analysis had low statistical support (Supplementary Figure 2). This result was also confirmed by the absence of significant genetic differentiation (F_{ST}) between mitogenomes of social forms in three out of four populations (Finges = 0.06, 95% CI [-0.07, 0.22]; Derborence = 0.08, 95% CI [-0.02, 0.18]; Sallanches = 0.62, 95% CI [0.28, 0.82]; Les Bussets = 0.06, 95% CI [-0.05, 0.14]).

Discussion

The impact of mating system and sex-biased gene flow on the maintenance of genetically determined complex phenotype is still poorly understood. In ants, it is commonly assumed that males disperse, whereas female are more philopatric (Hölldobler & Wilson 1990). In addition, alternative social forms may present distinct dispersal strategies (Bourke & Franks 1995; Zahnd *et al.* 2021). In the socially polymorphic ant *Formica selysi*, mature monogyne colonies are exclusively headed by Sm/Sm queens mated with Sm males (Avril *et al.* 2019a). Evidence of mature field colonies headed by a Sm/Sm queen mated with a Sp male is lacking, even if this cross is observed in nature (Fontcuberta *et al.* 2021) and produce viable offspring in the lab (Avril *et al.* 2019b). In addition, polygyne colonies never produce Sm/Sm females and Sm males due to a maternal effect killing (Avril *et al.* 2020).

Table 1. Mitochondrial variation across Hymenoptera

Species	Family	Number of variable sites	Fragment length (bp)	Number of samples (and populations)	Source
Formica selysi	Formicidae	40	16,752	48 (4)	This study
Formica paralugubris	Formicidae	3	5,165	11 (7)	Holzer *et al.* (2009)
Formica exsecta	Formicidae	61	1,500	86 (17)	Goropashnaya *et al.* (2007)
Formica fusca	Formicidae	25	555	93 (12)	Johansson *et al.* (2018)
Cataglyphis hispanica	Formicidae	116	520	23 (14)	Darras & Aron (2015)
Oecophylla smaragdina	Formicidae	89	639	72 (67)	Rahman *et al.* (2021)
Apis mellifera capsensis	Apidae	124	13,207	19 (19)	Eimanifar *et al.* (2018)
Vespula vulgaris	Vespidae	26	1,872	70 (4)	Dobelmann *et al.* (2019)
Cleruchoides noackae	Mymaridae	23	589	126 (4)	Nadel *et al.* (2012)
Protaphidius nawaii	Braconidae	80	422	41 (7)	Yamamoto *et al.* (2020)
Trigonaspis synaspis	Cynipidae	42	433	166 (21)	Mutun & Atay (2015)

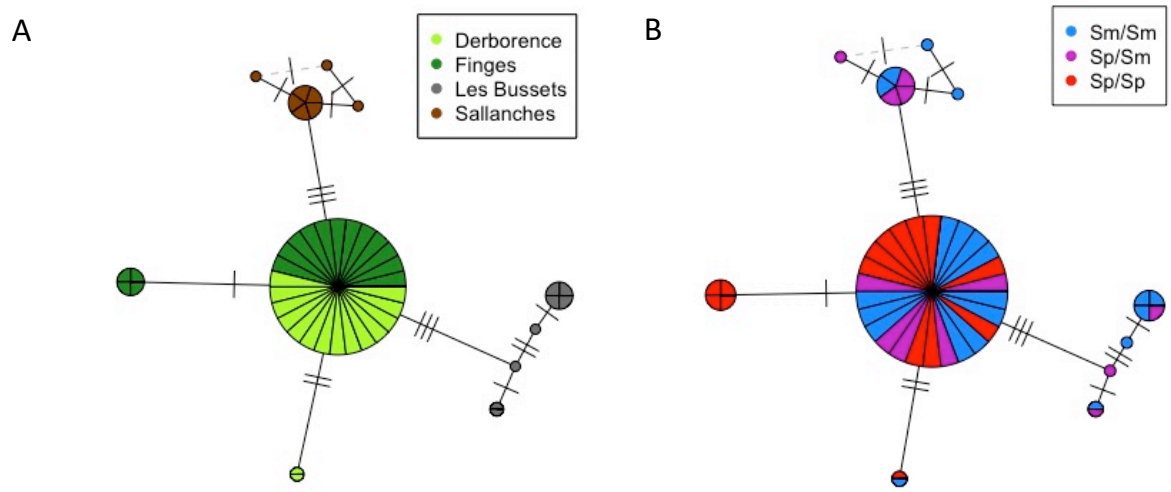


Figure 3. haplotype network based on 48 complete mitogenomes from four *Formica selysi* populations. Mitogenomes are colored according to geographical origin (A) and social origin (B). They cluster by geographical origin (A), rather than by social form (B). Dashed lines represent alternative links between haplotypes.

Here, we take advantage of whole genome sequencing to assess if female-mediated gene flow occurs between *F. selysi* social forms, and assess whether cyto-nuclear incompatibilies might be involved in the maternal effect killing in *F. selysi*. The mitogenomes of *F. selysi* present low genetic variation compared to other Hymenoptera. There is no or little genetic differentiation between social forms at the mitochondrial level, indicating at least occasional female-mediated gene flow. Overall, these results provide evidence that cyto-nuclear incompatibilities likely do not play a role in the maternal effect killing in *F. selysi*, as the supergene is not consistently co-transmitted with the cytoplasm. In addition, the lack of mitochondrial genetic differentiation tends to indicate that Sm/Sm queens mated with Sp males produce mature colonies in the field, that likely transition to polygyne colonies as proposed by Fontcuberta et al. (2021). The frequency of this phenomenon cannot be inferred by the present study, as even sporadic transition events suffice to avoid the accumulation of mitochondrial differentiation.

Maternal effect killers can operate through a modification-rescue system, such as a toxin and an antidote (Burt & Trivers 2006; Werren 2011). A possible mechanism for such a process would be to have the toxin in the supergene and the antidote in the cytoplasm or *vice versa*. Our results indicate that the maternal effect killing is likely not due to cyto-nuclear incompatibilities, as supergenes and mitochondria, and therefore cytoplasm, are not co-transmitted in our study system. For the same reason, our results also suggest that the maternal effect killing does not imply *Wolbachia* induced incompatibility (Beckmann *et al.* 2019). The mechanism underpinning transmission ratio distortion in *F. selysi* is still to be discovered.

Such lack of genetic differentiation suggests that Sm/Sm queens mated with Sp males produce mature colonies in the field, that likely transition to polygyne colonies as proposed by Fontcuberta et al. (2021). As a selfish genetic element, the Sp haploype bias its own transmission ratio (Avril *et al.* 2020). The result of this study suggests that, in addition to this phenomenon, the Sp haploype would also trigger the transition of colonies headed by an Sm/Sm queen into polygyne colony. Indeed, Sm/Sm queens mated with Sp males will produce Sp/Sm workers and queens, that may adopt additional queens carrying the Sp haplotype, transitioning to a multiple-queen colony. This indicates that polygyne colonies could spread by converting monogyne colonies into polygyne colonies via Sp males. This conversion would lead to female-mediated gene flow from the monogyne to the polygyne form and to the introgression of the monogyne mitochondrial haplotype to the polygyne form.

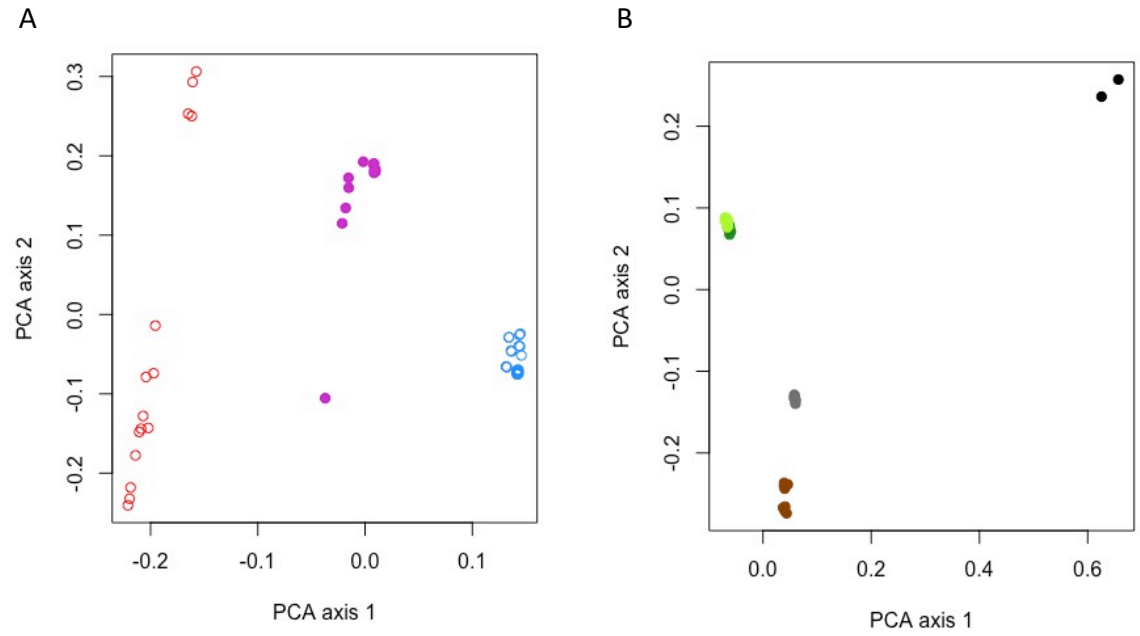
Furthermore, as monogyne queens have been shown to have better long-range colonization abilities (Zahnd *et al.* 2021), the Sp haplotype could favor its own propagation by using monogyne queens to reach new habitats and populations (Fontcuberta *et al.* 2021). A similar process has been hypothesized in the fire ant *Solenopsis invicta* (Ross & Shoemaker 1997) and has been demonstrated in mice (Runge & Lindholm 2018), suggesting that elevated dispersal is frequently linked to distorter supergene haplotypes.

In conclusion, our results provide novel insights into the mating system and dispersal of a species harboring a supergene-mediated complex polymorphism. Specifically, our study reveals that supergene and mitochondria are unlinked in the Alpine silver ant, suggesting the occasional introgression of the monogyne mitochondrial haplotype to the polygyne social form. The most likely scenario for such maternal gene flow is that monogyne queens mate with polygyne males, and establish colonies that become polygyne and produce Sp/Sm polygyne queens. Additional experiments on the mechanism underpinning the maternal effect killing are needed. Overall, our study demonstrates the power of comparing autosomal and sex-linked markers to gain a better understanding life-cycles and mating systems.

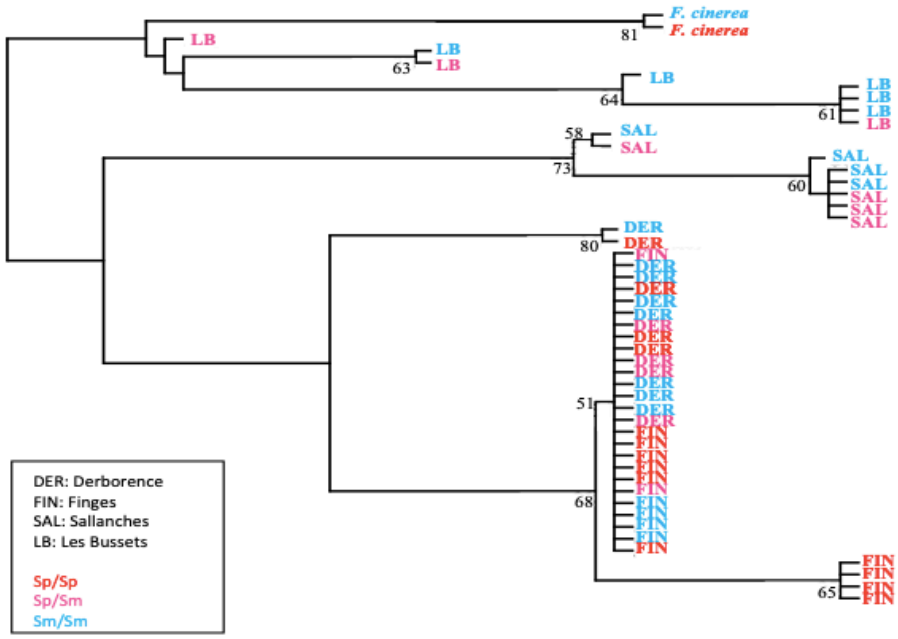
Acknowledgments

We thank Tristan Cumer and Ana Paula Machado for discussion regarding the analyses and Hélène Boulain, Ana Paula Machado and Robin Séchaud for discussion on the manuscript.

Supplementary materials



Supplementary Figure 1. PCA of the supergene (A, using 168,072 SNPs) and nuclear scaffolded SNPs excluding the supergene (B, using 869,587 SNPs) of 48 workers of *Formica selysi*. The panel A is showing three well-defined clusters associated with social organization. Each dot corresponds to one individual. The three well-defined clusters on the first axis correspond to Sm/Sm individuals (in blue), Sm/Sp individuals (in purple) and Sp/Sp individuals (in red). Solid symbols indicate strong heterozygosity ($F_{IS} < 0$) at the supergene, as expected for Sm/Sp heterozygotes. In plot B, the individuals (represented by dots) cluster according to species and populations (*F. cinerea* individuals are in black, *F. selysi* ones from Sallanches in brown, from Les Bussets in grey, from Finges in dark green and from Derborences in light green).



Supplementary Figure 2. Maximum-likelihood tree based on the complete mitogenomes. Numbers at nodes indicate bootstrap values for 1,000 pseudo-replicates (mean branch length = 1.25 x 10^{-5}). Mitogenomes cluster by geographical origin (indicated by their labels) and not by social genotype (indicated by colors).

General discussion

Hybridization between two ant species

Hybridization provides insights into the process of speciation, as it allows to describe mechanisms of pre- and post-mating isolation. The first part of this thesis describes a hybrid zone between two ant species, *Formica selysi* and *Formica cinerea,* and the pre- and post-mating mechanisms limiting gene flow between them. I first characterized the zone and investigated the frequency of hybrid workers, the presence of hybrid males and females across populations, and whether hybridization is influenced by colony social organization. I found that the hybrid zone between these two species exhibits a mosaic structure, with some populations formed exclusively by *F. selysi* or by *F. cinerea*, and others entirely by hybrids or by a mix of pure species and of hybrid colonies. Across all populations, the distribution of hybrids was asymmetric and skewed towards *F. cinerea*. The occurrence of backcrossed individuals indicates that fertile hybrid queens and/or males are produced. In addition, F1 hybrids generally have *F. cinerea* mothers and *F. selysi* fathers, as 97% of these hybrids had *F. cinerea* mitochondrial haplotype. Asymetric hybridization is common and has been identified in many species (e.g. Johnson *et al.* 2015; Kenney & Sweigart 2016; Sardell & Uy 2016), and in this case, it does not seem to depend on mate choice nor on the survival of F1 hybrids but could be associated with behavioral interactions between the two parental species and hyrbids (Chapter 2). Asymmetric hybridization can also result from hybrid zone movements (Barton & Hewitt 1985; Excoffier *et al.* 2009), with one of the species expending its range at the expense of the other, or from species differences in dispersal (Currat *et al.* 2008). In our case, the observed pattern could be due to a higher dispersal of *F. selysi* males compared to *F. cinerea* males, that will mate with *F. cinerea* females. Additional data on the stability of this hybrid zone but also

further investigation of the life cycle of both species will provide insights into this process, by understanding of the demographic processes underlying hybrid zones in social insects.

In Chapter 1, I found no evidence that the presence of genetically determined social forms influences hybridization. Even though the two species are socially polymorphic (Rosset & Chapuisat 2007; Purcell *et al.* 2014b; Brelsford *et al.* 2020), there were hybrids in both monogyne and polygyne colonies, suggesting that the supergene-mediated alternative social forms do not prevent hybridization. More studies should try to understand whether supergene-mediated complex phenotypes play any role in species delimitation, a topic that has so far been not extensively studied.

In Chapter 2, I investigated potential evolutionary and proximate mechanisms delimiting species in the hybrid zone described in Chapter 1 (Purcell *et al.* 2016). Altogether, I found no apparent cost of hybridization. Specifically, there were no signs of genetic incompatibilities between species, as hybrids of all sexes and castes were produced in the field and F1 hybrid workers had similar viability compared to non-hybrid workers. Despite the apparent absence of costs, assortative mating limits gene flow between species, as queens and males strongly prefered to mate with conspecifics. I did not find other mechanisms preventing gene flow, as there were no signs of temporal segregation in the production of young queens and males between the two species. The apparent absence of strong hybridizing costs, and therefore of the ultimate forces leading to the observed assortative mating, is intriguing. Proximatly, the mechanisms accounting for this mate preference may be differences in cuticular hydrocarbons, as workers of each species have different cuticular hydrocarbons and recognized conspecifics (Purcell *et al.* 2016 – Chapter 1 and Chapter 2). It has been shown that hydrocarbon cues can diverge between species as a result of genetic drift, local adaptation or sexual selection, and these cues affect mate choice and increase reproductive isolation in insect species (Blows &

Allan 1998; Schwander *et al.* 2013; Maroja *et al.* 2014), However, some costs were not measured here, like differences in colony mortality or lower fitness of hybrid sexuals. These costs could be driving and re-inforcing the evolution of assortative mating.

Overall, this first part descibed a new ant hybrid zone and sheds light on the underpinning isolation mechanisms involved. It proposed that well-developped nestmate recognition system of ants could play a role in the evolution of assortative mating, even in the absence of large hybridization costs. Together, it provided new insights into social insects' speciation.

Maintenance of a supergene-mediated polymorphism

The results of Chapter 3 (Zahnd *et al.* 2021) suggest that spatially-varying selection for competition- and dispersal-related traits may play a role in the coexistence of social forms. The two social forms occupy distinct habitats, with monogyne colonies being more frequent in less populated and more disconnected habitat patches, whereas polygyne colonies were abundant in saturated and more connected habitats. Interestingly, another case of spatially-varying selection has been suggested to contribute to the maintenance of a supergene polymorphism involved in mimetic wing coloration in the butterfly *Heliconius numata* (Joron *et al.* 1999). Investigating the role of temporal and spatially-varying selection in the maintenance of social polymorphism could thus be of great importance, both theoretically and empirically. One could predict that spatially-varying selection in the carrying capacities of patches, for exemple due to regular disturbance, facilitates the invasion of polygyne populations by the "colonizer" monogyne social form (McPeek & Holt 1992). Overall, these results open promising opportunities to study the role of ecological factors, spatial distribution and dispersal in the maintenance of social polymorphism in *F. selysi*.

The long-term maintenance of a supergene-controlling social polymorphism in *F. selysi* over 20-40 mya likely results from a combination of several forces (Brelsford *et al.* 2020). Indeed,

the polygyne social form is associated with a selfish drive of the Sp haplotype through maternal killing (Avril *et al.* 2020), which likely does not result from cyto-nuclear incompatibilities (Chapter 4). Theoretical modelling predicts that this would lead to the fixation of the polygyne social form under many conditions, even in presence of heterozygote advantage (Ghaseminejad *et al.,* unpublished). This model suggests that the maintenance of a stable polymorphism under this maternal killing requires very specific conditions, like strong assortative mating by social form, so that monogyne females mate mostly with monogyne males, and large differences in fitness between supergene genotypes. Recent studies in *F. selysi* may support these conditions, with strong assortative mating (Avril *et al.* 2019a; Fontcuberta *et al.* 2021) and a difference in dispersal and colonization abilities between the social forms (Zahnd *et al.* 2021 – Chapter 3).

The maternal effect killing affects gene flow between the social forms (Avril *et al.* 2020). Indeed, unidirectional gene flow from the monogyne to the polygyne social form has been inferred from population genetic data in *F. selysi* (Avril *et al.* 2019a) and in two other socially polymorphic ant species, *Solenopsis invicta* (Shoemaker & Ross 1996; Fritz *et al.* 2006) and *S. germinata* (Lacy *et al.* 2019), all three having a selfish drive of the polygyne haplotype. Such gene flow between social forms may be mediated by males of monogyne origin mating with females of polygyne origin (Ross & Keller 1995; Shoemaker & Ross 1996; Fritz *et al.* 2006; Avril *et al.* 2019a; Lacy *et al.* 2019). In addition, we have not found any behaviour and genetic barriers between Sm/Sm queens of monogyne origin and Sp males of polygyne origin (Avril *et al.* 2019b – Appendix 1). The results of Chapter 4 suggest that female-mediated gene flow from the monogyne to the polygyne form of *F. selysi* likely results from monogyne queens mating with polygyne males that found colonies that become polygyne by producing Sp/Sm queens. Together, this approach demonstrates the power of comparing autosomal and sex-linked markers to gain insights into complex life cycle and calls for a wider use of such comparisons.

Overall, these results shed the light on the mechanisms and evolutionary forces contributing to balance the frequency of the two haplotyes of a supergene controlling social organization. In particular, the role of spatially-varying selection as well as female-mediated gene flow as factors that may contribute to the maintenance of a supergene-mediated polymorphism.

Future directions

Supergenes controlling social organization appear three times independently in the evolutionary history of ants (Wang *et al.* 2013; Purcell *et al.* 2014b; Braims 2015; Brelsford *et al.* 2020), suggesting that this type of genomic structure may be widespread in ants. As other *Formica* species harbor an homologous version of the supergene of *F. selysi* (Brelsford *et al.* 2020), this would offer the possibility for comparative studies, both within and between genera. This type of approach has given promising results in other supergene systems, as in *Solenopsis* ants and in *Heliconius* butterflies (Joron *et al.* 2006).

The recent characterization of a hybrid zone between *F. selysi* and *F. cinerea* and the description of factors limiting hybridization would offer the opportunity to investigate the genomic of hybridization between two species bearing a supergene controlling social organization. Supergene introgression was highlighted in the *Heliconius* and in the *Solenopsis* species complexes (Jay *et al.* 2018; Stolle *et al.* 2021), revealing the high impact of hybridization on the evolutionary trajectory of supergene-mediated complex phenotype. We do not yet know whether such supergene introgression has occured across species belonging to the *Formica* genus. This genus is species rich, with many monogyne, polygyne and polymorphic species, as well as hybrid zones. The supergene contains small, disjunct clusters of conserved trans-pecies SNPs, suggesting an ancient origin, but also rare recombination (Brelsford *et al.* 2020). Further comparative studies across the *Formica* species shall provide new light on the evolutionary history of supergenes.

Appendix I

No mate preference associated with the supergene controlling social organization in Alpine silver ants

Amaury Avril, **Sacha Zahnd**, Jelisaveta Djordjevic, and Michel Chapuisat

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Abstract

Disassortative mating is a powerful mechanism stabilizing polymorphisms at sex chromosomes and other supergenes. The Alpine silver ant, *Formica selysi*, has two forms of social organization – single-queen and multiple-queen colonies – determined by alternate haplotypes at a large supergene. Here, we explore whether mate preference contributes to the maintenance of the genetic polymorphism at the social supergene. With mate choice experiments, we found that females and males mated randomly with respect to social form. Moreover, queens were able to produce offspring irrespective of whether they had mated with a male from the same or the alternative social form. Yet, females originating from single-queen colonies were more fertile, suggesting that they may be more successful at independent colony founding. We conclude that the pattern of asymmetric assortative mating documented from mature *F. selysi* colonies in the field is not caused by mate preferences or major genetic incompatibilities between social forms. More generally, we found no evidence that disassortative mate preference contributes to the maintenance of polymorphism at this supergene controlling ant social organization.

Introduction

The genetic basis of behavioral variation and the maintenance of adaptive diversity within populations are central questions in evolutionary biology. Recently, supergenes controlling complex social phenotypes have been discovered in ants and birds (Wang *et al.* 2013; Purcell *et al.* 2014b; Küpper *et al.* 2016; Tuttle *et al.* 2016). Supergenes are large genomic region of suppressed recombination. Because of tight linkage, alternate haplotypes of supergenes harbor clusters of co-adapted alleles that are transmitted together and cause coordinated variation in multiple traits, including morphology, physiology and behavior (Schwander *et al.* 2014; Thompson & Jiggins 2014). This unusual genomic architecture raises immediate questions on the origin, evolution and maintenance of supergenes.

The long-term persistence of polymorphic supergenes indicates that they are subject to balancing selection, generally through some form of heterozygote advantage or frequency-dependent selection (Llaurens *et al.* 2017; Wellenreuther & Bernatchez 2018). In several cases, the mutant haplotype is a recessive lethal, while heterozygotes have a fitness advantage (e.g. fire ant, ruff; Wang *et al.* 2013a; Küpper *et al.* 2016). Spatial or temporal variation in selection can also contribute to stabilize polymorphisms (e.g. mimetic butterfly, land snail; Joron *et al.* 2011; Richards *et al.* 2013). Last, supergenes controlling alternative reproductive phenotypes can be balanced by disassortative mating.

Disassortative mating, a process whereby mates are less similar than expected by chance, is a powerful mechanism balancing polymorphism through frequency-dependent selection, because the rarer type gains a reproductive advantage over the more frequent type (Fisher 1930). Obligate disassortative mating maintains polymorphism at sex chromosomes and mating type chromosomes (Charlesworth & Mank 2010; Beukeboom & Perrin 2014; Branco *et al.* 2018). Disassortative mating also stabilizes supergenes that do not determine sex in plants and animals.

The common primrose, *Primula vulgaris,* has heteromorphic flowers that have either long style and low anthers, or short style and high anthers (heterostyly). Obligate out-crossing with the alternative flower morph balances the frequencies of alternate allelic variants at the supergene controlling heterostyly (Li *et al.* 2016). In the white-throated sparrow, *Zonotrichia albicolis*, near-perfect disassortative mating between alternative morphs leads to a balanced polymorphism at a supergene controlling plumage color and social behavior (Tuttle *et al.* 2016; Hedrick *et al.* 2018; Sun *et al.* 2018). Finally, mate preference for morphs with alternative wing-pattern contributes to the maintenance of polymorphism at a supergene regulating Müllerian mimicry in *Heliconius numata* (Chouteau *et al.* 2017). In that mimetic butterfly, disassortative mate preference prevents the fixation of the morph that is most abundant and best protected from predators.

In the Alpine silver ant, *Formica selysi*, a large supergene with two haplotypes, Sm and Sp, is associated with colony social organization (Purcell *et al.* 2014b; Avril *et al.* 2019a). The species is socially polymorphic. Within the same populations, "monogynous" colonies harbor a single queen that monopolizes reproduction, while "polygynous" colonies contain multiple queens sharing reproduction (Chapuisat *et al.* 2004; Purcell & Chapuisat 2013; Purcell *et al.* 2015). Queens, workers and winged males from polygynous colonies carry at least one copy of the Sp haplotype. Specifically, all queens and workers from polygynous colonies have the supergene genotypes Sp/Sm or Sp/Sp, while males produced by polygynous colonies have the supergene haplotype Sp (male ants are haploid; Purcell *et al.* 2014c; Avril *et al.* 2019a). In contrast, all individuals from monogynous colonies lack the Sp haplotype and carry exclusively the Sm haplotype (all queens and workers have the supergene genotype Sm/Sm, and males the haplotype Sm; Purcell *et al.* 2014c; Avril *et al.* 2019a). An unusual feature of the *F. selysi* supergene is that both homozygotes are viable. The mechanisms contributing to maintain the polymorphism at this social supergene are not yet known.

In principle, disassortative mate preference might contribute to balance the polymorphism at the supergene controlling social organization in *F. selysi*. Yet, genetic evidence from mature colonies in the field suggest a pattern of asymmetric assortative mating (Avril *et al.* 2019a). In monogynous colonies, all queens had the Sm/Sm genotype and were mated with males having the Sm haplotype. In contrast, queens heading polygynous colonies were mated with Sp males or Sm males, the latter accounting for 22.9% of the matings (Avril *et al.* 2019a). Polygynous colonies do not produce Sm males and Sm/Sm females because the Sp haplotype is a maternal effect killer. Specifically, eggs from heterozygous queens that did not inherit Sp failed to hatch (Avril, Purcell, Béniguel, & Chapuisat, unpublished results). Hence, Sp males are exclusively produced by polygynous colonies and Sm males by monogynous colonies (Purcell *et al.* 2014b; Avril *et al.* 2019a). Overall, all queens heading mature monogynous colonies had mated assortatively, while a fraction of queens from polygynous colonies had mated disassortatively, with males originating from the alternative social form.

The causes for the mating pattern documented in the field remain elusive (Avril *et al.* 2019a). Indeed, the degree of disassortative mating in mature colonies depends on multiple factors, including mate availability, mate preference, and genetic compatibilities. Because sex ratio, productivity and probably dispersal vary greatly between monogyous and polygynous colonies, queens originating from monogynous colonies may encounter primarily Sm males when mating in nuptial swarms, while queens originating from polygynous colonies may encounter primarily Sp males when mating close to their natal nest (Rosset & Chapuisat 2006, 2007). Disassortative mate preference by polygynous queens could thus favor locally rare Sm males over Sp males, and such rare male advantage could contribute to balance the polymorphism. Conversely, assortative mate preference tends to restrict gene flow between social forms and might even lead to speciation, a process that is not supported by the absence of genetic differentiation between social forms at loci outside of the supergene (Purcell & Chapuisat 2013; Purcell *et al.*

2014b; Avril *et al.* 2019a). Overall, it is of interest to investigate if mate preferences or genetic incompatibilities between social forms play a role in the dynamics of this unusual genetic system.

With mate choice experiments, we assessed whether mate preferences or genetic incompatibilities between social forms explain the pattern of asymmetric assortative mating observed in mature field colonies. This would be the case if (i) queens of polygynous origin readily mate with males of monogynous origin, while (ii) queens of monogynous origin do not mate with males of polygynous origin or (iii) queens of monogynous origin mated to males of polygynous origin fail to produce offspring. At a more fundamental level, we test if disassortative mate preference by queens of polygynous origin contributes to balance the polymorphism at this supergene controlling ant social organization.

Materials and Methods

Sampling

Virgin queens (= young winged females) and males of *Formica selysi* were collected in central Valais, Switzerland, in summer 2015 from 12 colonies in Finges (7°36'30" E, 4°18'30" N, altitude: 565m) and 30 colonies in Derborence (7°12'56" E, 46°16'50" N, altitude: 1450m). The social organization of each colony had been previously determined based on direct observations of queens that warm up under stones in early spring, microsatellite genotyping, RAD-seq genotyping and PCR-RFLP genotyping of SNPs diagnostic for social form (Purcell & Chapuisat 2013; Avril *et al.* 2019a). Most colonies of *F. selysi* specialize in the production of one sex (Rosset & Chapuisat 2006). Virgin queens or males from each colony were kept separate in small plastic boxes, with workers from the same parent colony, at 24°C and under a relative humidity of 50% (Avril *et al.* 2019a). The ants had access to water and *ad libitum* food.

Mate preferences

With mate choice experiments, we examined whether queens and males prefer to mate with partners of the same or the alternative social form. In each trial, a single virgin queen and four males, two from each social form, had the opportunity to mate. The queen and males originated from different colonies of the same population. Males of alternative social forms were color-marked, with colors randomized across trials. Queens were unmarked. The queen and males were transferred to a mating arena consisting of a box covered by a net (35 × 22 × 15 cm). Each box had a masked label, so that during the mate choice experiment the observers were kept blind with respect to the social origin of queens and males. For the mating trials, the boxes were placed outdoor, in the morning and in daylight, which elicits flying and mating behavior (Reber *et al.* 2010). We monitored the behavior of queens and males until mating, if any, or up to 30 minutes otherwise. Queens and males that did not mate in the first trial were returned to their lab colonies and used in at most one other trial.

Genetic incompatibilities

To detect potential genetic incompatibilities between social forms, we assessed the success of each mated queen at founding incipient colonies and producing brood. Immediately after mating, the queen was isolated in a glass test tube labelled with a unique number, so that the subsequent observers were kept blind to the social origin of the queen and her mate. Each tube had water blocked by cotton wool at the bottom and was wrapped in aluminum foil for darkness, which mimics independent claustral colony founding by solitary queens (Brütsch *et al.* 2017). In each incipient colony the number of eggs, larvae, cocoons and workers, as well as the status of the queen (dead or alive), were recorded every other day over 80 days after mating.

Statistical analyses

Mate preferences and queen mating propensity were analyzed with generalized mixed effect models (GLMM), using a binomial error distribution. For mate preferences, we built a model in which the response variable was the social origin of the queen's mate (monogynous or polygynous, respectively). The social origin of the queen was included as fixed effect. Random effects comprised colony of origin of queen and males, color marks, trial date, and whether the queen or males did mate in the first or second trial, if any. A Wald test on the intercept was used to detect significant departure from random mating. To estimate the power of this analysis, we simulated 1,000 datasets with 20% of disassortative mating, a degree of deviation comparable to the ones documented from field colonies (Avril *et al.* 2019a). With our sample scheme, the power to detect deviations from random mating of this magnitude was 92.3% and 77.3% for queens of monogynous and polygynous origin, respectively. For the mating propensity of queens, the response variable was the mating status of the queen (mated or not) at the end of the trial. The queen social origin was included as fixed effect, while random effects comprised the trial date and whether the queen or males did mate in the first or second trial.

We explored whether genetic incompatibilities between social forms affected the success of solitary queens at founding incipient colonies, as well as brood production in successful colonies. For the success at founding incipient colonies, we used a GLMM with a binomial error distribution. Colony success, i.e. whether the queen had survived until the end of the experiment and succeeded in producing workers, was the binomial response variable. Queen social origin, male social origin and the interaction between the two factors were included as fixed effects. Random effects comprised the trial date and whether the queen or males did mate in the first or second trial. For brood production, we used a generalized additive mixed model (GAMM), which can model non-linear time series data (Zuur *et al.* 2009). The response variable was the number of brood items (eggs, larvae, cocoons and workers) per queen, across

fertile queens that survived until the end of the experiment. Queen social origin, male social origin and the interaction between the two factors were included as fixed effects. The number of days after mating was used as the smoothing covariate. Random effects comprised queen identity and whether the queen or males did mate in the first or second trial. All statistics were performed with the R statistical package v. 3.3.2 (R Core Team 2015). GAMM and GLMM models were built using the 'mgcv' package v1.8 (Wood 2011) and the 'lme4' package v1.1 (Bates *et al.* 2015), respectively.

Table 1. Success of solitary queens at founding incipient colonies, estimated as the proportion of incipient colonies in which the queen survived until the end of the experiment and succeeded in producing workers. The number of colonies monitored is indicated between parentheses

Mated to	Queen of monogynous origin	Queen of polygynous origin
Male of monogynous origin	0.71 (24)	0.60 (10)
Male of polygynous origin	0.68 (22)	0.43 (14)

Results

Mate preferences

In mate choice experiments involving a virgin queen and two males from each social form, mating occurred randomly with respect to social form. No significant mate preference was detected for queens of monogynous origin (Figure 1; GLMM binomial, $z_1 = -0.25$, $P = 0.81$), nor for queens of polygynous origin (Figure 1; GLMM binomial, $z_1 = 0.78$, $P = 0.43$). However, queens of monogynous origin were more likely to mate than queens of polygynous origin (Figure 1; mating occurred for 74.2 % of 62 queens of monogynous origin and 40.7 % of 59 queens of polygynous origin, respectively; GLMM binomial, $\chi^2_1 = 14.1$, $P < 0.001$).

Genetic incompatibilities

The success of incipient colonies did not depend on whether the founding queen had mated with a male from the same or the alternative social form (Table 1; GLMM, $\chi^2_1 = 0.05$, $P = 0.81$) and was not influenced by male social origin (Table 1; GLMM, $\chi^2_1 = 0.43$, $P = 0.51$). Colony success rate tended to be higher for queens of monogynous origin than for queens of polygynous origin, but the difference was not statistically significant (Table 1; GLMM; $\chi^2_1 = 3.0$, $P = 0.08$).

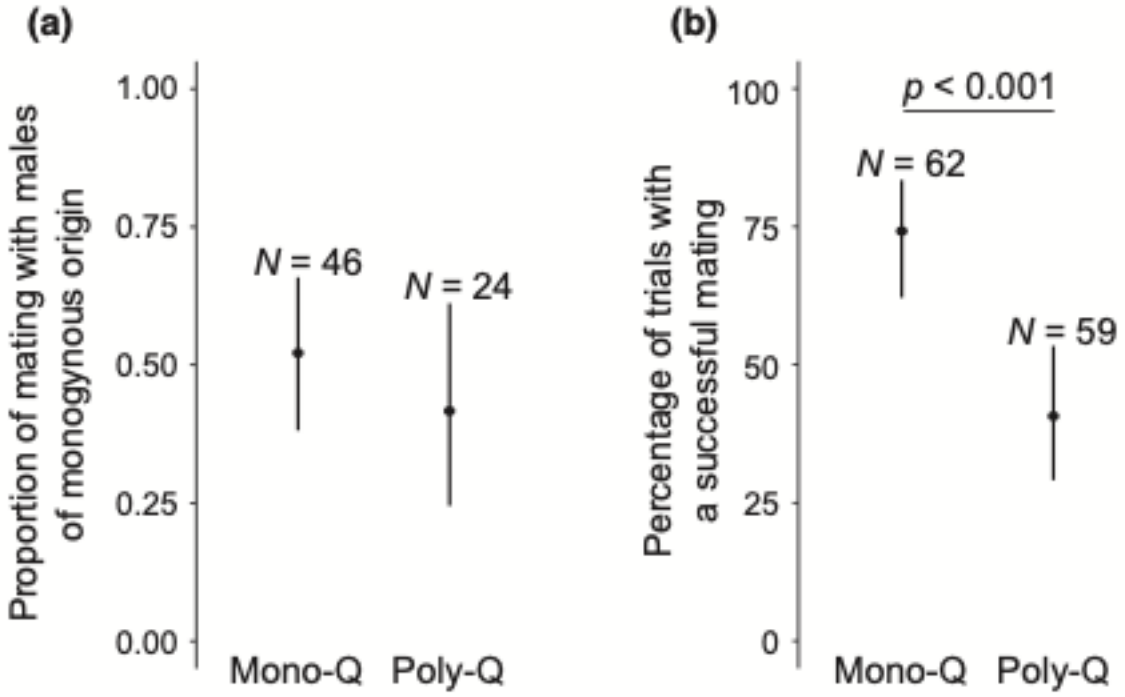

Figure 1. Mate choice experiments with *F. selysi* queens of monogynous origin (Mono-Q) and polygynous origin (Poly-Q), respectively. Each queen was presented with two males of each social form. (A) Mate preference. Frequency of mating occurring with males of monogynous origin. Bars indicate the binomial 95% confidence interval around the mean. The total number of matings is indicated above each bar. (B) Mating propensity. Percentage of trials in which the queen mated. Bars indicate the binomial 95% confidence interval around the mean. The total number of trials is indicated above each bar.

Brood production in successful incipient colonies was not influenced by whether the founding queen had mated with a male of the same or the alternative social origin (Figure 2; Interaction between social origins of queens and their mates, for eggs: $F_{1,1} = 0.72$, $P = 0.40$; Larvae: $F_{1,1} =$

1.03, $P = 0.31$; Cocoons: $F_{1,1} = 0.58$, $P = 0.45$; Workers: $F_{1,1} = 0.92$, $P = 0.34$). Male social origin did not influence brood production by queens (Figure 2; Eggs: $F_{1,1} = 0.18$, $P = 0.67$; Larvae: $F_{1,1} = 1.63$, $P = 0.20$; Cocoons: $F_{1,1} = 0.02$, $P = 0.88$; Workers: $F_{1,1} = 0.70$, $P = 0.40$). In contrast, queen social origin had a strong effect on brood production, with queens of monogynous origin producing significantly more brood than queens of polygynous origin (Figure 2; Eggs: $F_{1,1} = 26.7$, $P < 0.0001$; Larvae: $F_{1,1} = 42.5$, $P < 0.0001$; Cocoons: $F_{1,1} = 44.5$, $P < 0.0001$; Workers: $F_{1,1} = 54.8$, $P < 0.0001$).

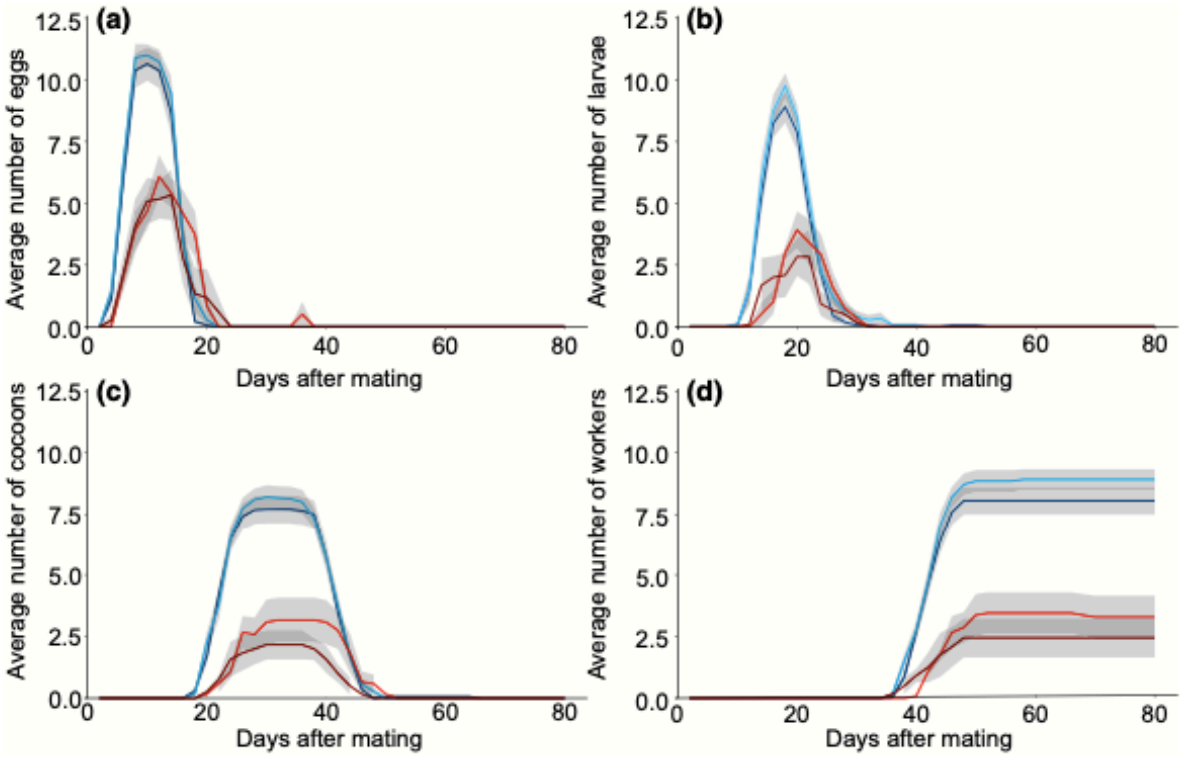

Figure 2. Brood production in successful colonies. Number of (A) eggs, (B) larvae, (C) cocoons, and (D) workers produced by queens of monogynous origin mated with males of monogynous origin (dark blue, N = 17), queens of monogynous origin mated with males of polygynous origin (light blue, N = 15), queens of polygynous origin mated with males of monogynous origin (light red, N = 6) and queens of polygynous origin mated with males of polygynous origin (dark red, N = 6), respectively. Lines and shaded areas depict the average number of brood items and the standard error of the mean, respectively

Discussion

A large non-recombining region with two haplotypes determines colony social organization in the Alpine silver ant, *F. selysi* (Purcell *et al.* 2014b). Females and males originating from monogynous colonies carry exclusively the Sm haplotype, while females and males originating from polygynous colonies have one or two copies of the Sp haplotype(Avril *et al.* 2019a). This polymorphism is shared across populations and appears stable(Chapuisat *et al.* 1997, 2004; Purcell & Chapuisat 2013; Purcell *et al.* 2015; Avril *et al.* 2019a), but so far the mechanism of balancing selection remain unclear. Disassortative mating maintains polymorphism at sex chromosomes and other supergenes (Tuttle *et al.* 2016; Chouteau *et al.* 2017; Llaurens *et al.* 2017; Hedrick *et al.* 2018). This prompted us to investigate if some degree of disassortative mate preference could contribute to the maintenance of the polymorphism at the social supergene of *F. selysi*.

In mate choice experiments, females and males of *F. selysi* mated at random with respect to their social origin. These behavioral data provide no support to the hypothesis that disassortative mate preference balances the polymorphism at the supergene controlling social organization. Disassortative mating is associated with lethal homozygosity of one haplotype in many sex chromosomes and in at least two supergenes controlling social phenotypes (Charlesworth & Mank 2010; Wang *et al.* 2013; Küpper *et al.* 2016). The absence of disassortative mate preference in *F. selysi* is consistent with the fact that homozygotes for both haplotypes are viable and that we detected no major genetic incompatibility within social forms.

Mate preference has not been investigated in fire ants, because their mating takes place high in the air and cannot be observed or manipulated in controlled conditions (e.g., Mikheyev 2003). Fire ant social organization is controlled by a supergene that evolved independently from the one of *F. selysi* (Wang *et al.* 2013; Purcell *et al.* 2014b). Fire ant queens in mature monogynous

and polygynous colonies had mated predominantly with males originating from the monogynous social form (Shoemaker & Ross 1996; Fritz *et al.* 2006; Lawson *et al.* 2012). This unusual mating pattern might be linked to the lower fertility of males carrying the supergene haplotype associated with polygyny (Lawson *et al.* 2012).

In our experiment, *F. selysi* queens from each social form were able to produce offspring, independently of whether they had mated with a male from the same or the alternative social form. Two lines of evidence indicate that there are no major genetic incompatibilities within or between social forms. First, the success of queens at founding incipient colonies did not depend on the social origin of their mates. Second, offspring production in successful colonies was independent of whether the queen had mated with a male of the same or the alternative social form. These results corroborate earlier findings based on a larger number of colonies (Reber *et al.* 2010). They also confirm that workers can develop into adults irrespective of their supergene genotype – at least some to the Sm/Sm, Sp/Sp and Sm/Sp offspring are viable (Purcell *et al.* 2014b; Avril *et al.* 2019a). Due to the small number of incipient colonies that produced brood, the power of this experiment was not sufficient to detect more subtle genotypic incompatibilities. There is strong selection for disassortative mate preference when genetically similar partners are incompatible, and conversely for assortative mate preference when genetically dissimilar partners are incompatible (Tregenza & Wedell 2000; Mays & Hill 2004). Overall, we detected no major genetic incompatibilities within or between social forms of *F. selysi,* based on a small number of crosses monitored during the early stages of colony development in protected laboratory conditions. Thus, such incompatibilities are unlikely to promote assortative or disassortative mating with respect to the social origin of queens and males.

Mate preference or genetic incompatibilities did not explain the fact that all queens in mature monogynous colonies had mated with males of monogynous origin, while 22.9% of the queens

in mature polygynous colonies had mated with males of monogynous origin (asymmetric assortative mating Avril *et al.* 2019a). In the mate choice experiments, queens showed no preference for males of monogynous origin and males of monogynous origin did not outperform males of polygynous origin. In particular, Sm/Sm queens did mate with Sp males and this cross produced viable offspring. Yet, Sm/Sp workers or Sm/Sm queens that had mated with Sp males have never been detected in mature monogynous field colonies, which are several years old (Purcell *et al.* 2014b; Avril *et al.* 2019a). It is possible that Sm/Sm queens do not encounter Sp males in the field, due to differences between social forms in the number, timing or dispersal behavior of queens and males(Rosset & Chapuisat 2006, 2007). Alternatively, when they age, incipient colonies founded by Sm/Sm queens that had mated with Sp males might be quickly converted into polygynous colonies headed by multiple Sp/Sm daughter queens.

Queens of alternative social forms showed some differences in their mating and reproductive strategies. Queens of monogynous origin were more likely to mate in our experimental settings that mimicked a mating flight, which suggests that they might be more prone to mate outside of their nests. More importantly, queens of monogynous origin produced three times as many brood than queens of polygynous origin, irrespective of the social origin of their mates. *F. selysi* queens of monogynous origin are slightly bigger than queens of polygynous origin (Rosset & Chapuisat 2007; Meunier & Chapuisat 2009), which may explain their higher productivity in experimental conditions mimicking independent claustral colony founding, without food and workers. In contrast, queens of polygynous origin may preferentially mate close to or within their natal nest and establish new nests with the help of workers. This shift in body size, dispersal and mode of colony founding is commonly associated with the transition to polygyny in ants(Hölldobler & Wilson 1977; Keller & Passera 1989). Consistent with more restricted dispersal and dependent colony founding by queens of polygynous origin, nestmates queens,

as well as queens and their mates, are significantly related in polygynous colonies (Avril *et al.* 2019a).

In summary, we found no evidence that disassortative mating contributes to stabilize the polymorphism at the social supergene of *F. selysi*. Moreover, mate preferences or strong genetic incompatibilities between social forms do not explain the pattern of asymmetric assortative mating observed in the field, which probably reflects differences in mate availability and colony development. Yet, queens of monogynous origin were more fertile than queens of polygynous origin, which is consistent with the hypothesis that queens of monogynous origin are more successful at independent colony founding. Differences between social forms in dispersal and mode of colony founding might play a key role in the maintenance of the polymorphism. A plausible scenario is that the monogynous form has higher success at colonizing novel habitat patches, while the polygynous form outperforms the monogynous form in old, saturated habitat patches (spatial heterogeneity in selection; Pedersen & Boomsma 1999b; Purcell *et al.* 2015). Heterozygote advantage might also contribute to stabilizing this genetic polymorphism controlling ant social organization.

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