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Insects and incest: field evidence for dangerous liaisons in a parasitoid wasp

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Abstract

1. Sib mating avoidance is a pervasive behaviour, which likely evolves in species that sare subject to inbreeding depression. Laboratory studies have provided elegant demonstrations, but small-scale bioassays often minimize the costs associated with mate finding and mate-choice and may for this reason produce spurious findings.
2. We inferred the mating behaviour of the parasitoid wasp *Venturia canescens* from the genetic analyses of natural populations. We used *V. canescens* as a model organism because in this species, laboratory experiments have shown that sib mating yields a 25% decrease in fertile offspring, and congruently, sib mating is partially avoided.
3. Our study consisted in genotyping 86 wild-caught males, 155 wild-caught-females and their 226 daughters at eighteen microsatellite loci. With these data, we were able to reconstruct the genotype of females' mate and estimate the relatedness of each mating pairs.
4. We found that the effective rate of sib mating does not differ from the probability that sibs encounter one another at random, which suggest a sib mating tolerance in this species. However, complementary lab experiments confirmed that kin discrimination exist in this species, with related pairs having a lower mating latency.
5. These results suggest that *V. canescens* tolerate sib mating in the field despite kin discrimination, and therefore call into question the common beliefs on inbreeding depression in species with single-locus complementary sex determination. This inbreeding tolerance also opens up the question of the maintenance of the kin discrimination in this species.

Key-words: Field assay**,** Inbreeding tolerance, Kin recognition, Parasitoid wasp, single-locus Complementary Sex Determination

Introduction

When inbreeding lowers fitness-related components such as survival and fertility, natural selection should favour behaviours preventing the reproduction of genetically-related individuals or mitigating harmful consequences (Pusey and Wolf, 1996; Keller and Waller, 2002; Angeloni et al., 2011). Various strategies have been evidenced in animals, including sib mating avoidance, dispersal (Greenwood et al., 1978; Szulkin and Sheldon, 2008), polyandry, extra-pair paternity, divorce (Hatchwell et al., 2000; Cornell and Tregenza, 2007; Cohas et al., 2008; Lardy et al., 2011; Reid et al., 2015; Duthie and Reid, 2016), and postcopulatory mechanisms such as the preferential use of sperm from unrelated males by inseminated females (Tregenza and Wedell, 2002; Bretman et al., 2004). Mate-choice is

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probably the most pervasive behaviour for sib mating avoidance (Pusey and Wolf, 1996). Altogether, these strategies are associated to costs in the form of decreased survival and energy expenditure (Bonte et al., 2012), loss of mating opportunities (Cooney and Bennett, 2000; O'Riain et al., 2000), or the disruption of favourable gene association (Bateson, 1982, 1983). Selection on behaviours underlying inbreeding avoidance should thus depend on inbreeding load, which scales the advantage of inbreeding avoidance and the costs of implementing adapted behavioural responses. Selection may also depend on inclusive benefits of inbreeding, which result from the increased representation of genes identical by descent in inbred offspring (Kokko and Rankin, 2006; Puurtinen, 2011; Duthie and Reid, 2016).

The balance between benefits and costs associated with inbreeding and inbreeding avoidance depends on population structure. For instance, population density constrains mate availability and consequent encounter rate, and may therefore influence mate choice (Kokko and Rankin, 2006; Duthie and Reid, 2016). In the lizard *Zootoca vivipara*, female choosiness is reduced when mate encounter rate decreases (Breedveld and Fitze, 2015). Similarly, genetically-related partners are less avoided in small and isolated populations of moose, with an amplified effect when the sex-ratio is female-biased (Herfindal et al., 2014). In the marsupial carnivore *Antechinus agilis*, habitat selection following natal dispersal is negatively correlated with the abundance and relatedness of females occupying habitats, which suggests a pervasive effect of inbreeding risk on dispersal (Banks and Lindenmayer, 2014).

Responses to inbreeding reflect the diversity of benefits and costs associated with inbreeding and inbreeding avoidance. Observations range from systematic inbreeding avoidance to inbreeding tolerance, or even, inbreeding preference (Szulkin et al., 2013). For example, inbreeding preference is observed in the cichlid fish *Pelvicachromis taeniatus*, where parental care occurs, with higher cooperation and investment in related pairs (Thünken et al., 2007), or in rodents such as *Marmota flavipentris*, despite the lower survival of inbred progeny (Olson et al., 2012). On the other hand, ring-tailed lemurs or mole rats living in socially structured groups systematically avoid inbreeding (Cooney and Bennett, 2000; Boulet et al., 2009).

Assessing inbreeding avoidance pattern, that is, the occurrence of inbreeding avoidance and the behavioural strategies implied, is a difficult task that requires the estimation of relatedness coefficients between actual and potential mates (Szulkin et al., 2013). This may explain why most field studies have been conducted on large species of mammals or birds, for which monitoring is much easier compared to small invertebrates (Cohas et al., 2008; Herfindal et al., 2014; Arct et al., 2015; Hardouin et al., 2015)). It is therefore no surprise that inbreeding avoidance patterns have been rarely documented in insects, especially in the wild (but see (Robinson et al., 2012) for a field assessment of optimal inbreeding in *Drosophila melanogaster*).

In this study, we take up this challenge by studying, in the field, a parasitoid wasp with a simple form of inbreeding depression. Many species of the order Hymenoptera have single-locus complementary sex determination (sl-CSD) whereby sex is determined by both ploidy and heterozygosity at a unique sex determination locus (Cook, 1993; van Wilgenburg et al., 2006; Heimpel and de Boer, 2008). Males develop from unfertilized haploid eggs whereas females develop from fertilized diploid eggs. Inbreeding depression arises for diploids that are homozygous at the sex locus: instead of developing normally into females, they turn into abnormal diploid males that are generally unviable or sterile (Cook, 1993; van Wilgenburg et al., 2006; Fauvergue et al., 2015). Whatever the diversity of sex alleles in the population, full sibs have 50% chance of sharing a common sex allele, and, should they mate, half of their diploid offspring develops into unfit males (Cook, 1993) . Hence, assuming females fertilize half of their eggs, sib mating results in 12.5% fewer offspring, on average. sl-CSD is thus a form of inbreeding depression underlined by overdominance, with no

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deleterious alleles at the sex locus but a strong heterozygous advantage (Charlesworth and Willis, 2009). The low fitness of diploid males should favour sib mating avoidance.

In the parasitoid wasp *Venturia canescens* Gravenhost (Hymenoptera: Ichneumonidae) single locus complementary sex determination (Beukeboom, 2001) has a negative impact on the fitness of both males (Chuine et al., 2015) and females (Vayssade, 2014). Consistently, mating success in no-choice bioassays decreases with increasing genetic relatedness (Metzger et al., 2010a; Chuine, 2014; Chuine et al., 2015). Circumstantial evidence suggest that females are the choosy sex, which makes sense in a species with a monoandrous/polygynous mating system (Beukeboom, 2001; Metzger et al., 2010b; Fauvergue et al., 2015).

In this study, we developed a population genetic approach based on microsatellite genotyping in order to assess inbreeding avoidance and underlying behaviours in field populations of *Venturia canescens*. To fulfil this objective, we first sampled two natural populations of *V. canescens* and compared the observed rate of sib mating to the probability of random encounter among genetically-related individuals. Under the hypothesis of sib mating avoidance, we expected the rate of sib mating to be smaller than the probability that relatives encounter at random. In a follow-up experiment, we further explored the effect of density and average relatedness on inbreeding avoidance, with the expectation that sib mating avoidance should decrease with decreasing density and increasing relatedness. Finally, we used the field data to test whether sex-biased dispersal could limit encounters among sibs in the field. We show that sib mating tolerance occurs in the wild, and propose an evolutionary scenario to explain why this result contrasts with earlier findings and prior expectations.

Materials and Methods

Biological model

Venturia canescens is a solitary endoparasitoid found in most circum-Mediterranean areas ((Salt, 1976). Females lays eggs in a large range of hosts found on various fruits (Salt, 1976; Driessen and Bernstein, 1999; Collet et al., 2016). Some hosts of *V. canescens*, like *Ectomyelois ceratoniae* have a scarce distribution, with a single larva developing in rare infested fruits (Driessen and Bernstein, 1999). Male search for females via a synergistic combination of volatile sex pheromones emitted by females and kairomones from hosts ((Amat et al., 2009; Metzger et al., 2010b). Immature development time and adult lifespan are both around three weeks (at 25°C), which yields overlapping generations. Adults are good dispersers, with flight velocity estimated at 0.2 $m.s^{-1}$ (Schneider et al., 2002; Amat et al., 2012).

Insect sampling in the field

Adult *V. canescens* were captured from June to September 2014, across 10 non-consecutive days. Two field sites located 300 km apart were sampled: Valence (N 44°58'21" E 4°55'39", composed of fruit trees in an orchard) and Nice (N 43°41'23" E 7°18'6", composed of carob trees in a semi-natural park). We captured only females In Valence and wasps of both sexes in Nice.

Two different types of traps were used: kairomone-baits for the capture of live females, and yellow sticky traps for males, which were generally found dead. Female traps were constituted of an open cup containing host larvae, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), along with used rearing medium (semolina). Saliva secreted by hosts larvae when feeding serves as a kairomone for female *V. canescens* (Corbet, 1971). Traps were hanged in trees (Metzger et al., 2008) and visited every 20 minutes to capture attracted females. For males, we used yellow sticky traps baited with extracts from females and hosts, a method that has already proved successful (Collet et al., 2016). Trap consisted of a 125 mm × 200 mm yellow sticky trap with a vial containing the extracts attached in its centre. As for females, traps were hanged within host trees, and all the *V. canescens* males that were found stuck on the traps were collected and

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conserved individually in 96% ethanol to preserve DNA. We captured 77 females and 86 males in Nice and 78 females in Valence (see Results and Table 1).

Insect rearing

The wild females trapped were brought back to the laboratory where they were handled in climatic chamber under constant conditions (24±1°C, 60±10% RH, DL 16:8) and fed with 50% water-diluted honey. Each female was allowed to lay eggs individually in *E. kuehniella* hosts for three days. As sexual and asexual strains coexist in *V. canescens* ((Beukeboom et al., 1999; Schneider et al., 2002), we used the presence of males among offspring as evidence for arrhenotoky. Mothers and their offspring were killed and preserved individually in 96% ethanol. Thelytokous individuals were discarded.

Genotyping

Genotyping was performed based on 18 microsatellites developed by (Collet et al., 2016) and (Mateo Leach et al., 2012) (See SEM-A for details on the genotyping and SEM-D for details on the number of microsatellites used). We genotyped all field-captured males and females (thereafter referred to as wild females) and up to three daughters per female (see SEM-B for a justification of the number of offspring genotyped). When there were fewer than three daughters per female (ca. 40%), all were genotyped.

We estimated polymorphism and linkage disequilibrium at each microsatellite loci for the wild females of Nice and Valence with the GENEPOP software version 4.3 (Raymond and Rousset, 1995). The frequency of null alleles was estimated with FREE NA software (Chapuis and Estoup, 2007). Population differentiation was estimated based on Wright's *F* estimator F_{st} with the FSTAT software version 2.9.3 (Goudet, 1995). Effective population sizes were estimated using the full likelihood method, assuming random mating, developed by (Wang, 2009), and implemented in COLONY software (Jones and Wang, 2010). As effective population sizes were small (< 80), we did not check for Hardy-Weinberg equilibrium. We also sought for diploid males as a proxy of consanguinity and of the number of alleles at *csd* locus. A male was considered diploid if heterozygous at one or more microsatellite locus, haploid otherwise (Collet et al., 2016).

Inference of father genotype from field data and estimation of partner relatedness

- Inference of mate's genotype

We inferred the genotype of wild females' mates based on the genotypes of wild females and their offspring. For this, we used the software COLONY, which reconstructs families based on haplodiploid genotypes (Jones and Wang, 2010). When alternative genotypes were proposed for a given female's mate, we selected the only compatible one on a case-by-case basis. We successfully inferred

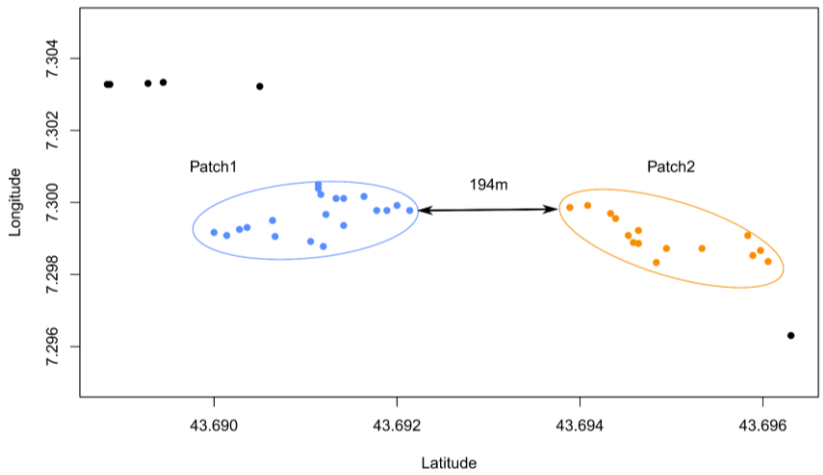

Figure 1 – Location of carob trees from the Nice field site where *Venturia canescens* were captured during the summer 2014. Each dot represents a tree. Axes are GPS coordinates. Blue dot represent trees of "Patch 1" and orange dots represent trees of "Patch 2" (see also Table 2). Six trees (black dots) were included in neither of the two patches, as they did not fit our definition of patches.

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51 mates' genotypes for the 54 mothers (see Results and Table 1).

- Estimation of relatedness between potential *versus* mated partners in the field

To assess if *V. canescens* females avoid mating with their relatives in the field, we compared the observed number of wild females mated with a related male to the number expected under the assumption of random mating. For this, we determined a "relatedness threshold" above which individuals were considered related. To determine this threshold, we simulated populations with similar allele frequencies at microsatellite loci to that of natural populations and a balanced sex ratio (se SEM-C for details on simulations). Simulations allowed to keep track of pedigrees, yielding a perfect knowledge of kinship for all potential mates. These were classified in three categories: full-sibs, half-sibs, or unrelated. Besides, we calculated the relatedness coefficient (r) for all female-male pairs from these simulated populations (software COANCESTRY;(Wang, 2011). This allowed to estimate the optimal relatedness threshold minimizing the number of wrong assignations (i.e., unrelated pairs assigned related based on the threshold, or vice-versa). We found $r_{crit} = 0.16$ (SEM-D, Fig. S2). Logically, this threshold is lower than the theoretical relatedness between a brother and a sister ($r_{\text{sister-brother}} = 0.25$; $r_{\text{brother-sister}} = 0.5$) and in the interval of relatedness for 1st degree cousins ($r = 0.125$ or $r = 0.1875$ depending on the cross). With this threshold, we expect to wrongly assign 11.4% of the relationships.

We applied this $r_{\text{crit}} = 0.16$ threshold to field data. First, we estimated the number of wild females that had been inseminated by a related male. Second, we generated a theoretical distribution of the number of couples with an $r \geq 0.16$ under the null hypothesis of random mating. For this, each of the 51 wild females from Nice, for which a mate genotype had been successfully inferred, was paired randomly with one of the 79 genotyped males, and we counted the number of pairs with $r \geq 0.16$. This calculation was repeated 1000 times to produce the null distribution. We finally compared the observed number of related pairs with the null distribution via a permutation test implemented in the function AS.RANDTEST from the ADE4 package in R software version 3.2.3 (Chessel et al., 2004; R Core Team, 2015). We also estimated the probability that a female encountered a related male in the field the day of her capture.

Sex-biased dispersal in the field

Sex-biased dispersal can decrease the burden of inbreeding depression by reducing encounters and subsequent mating among sibs. We thus assessed the dispersal ability of males and females between in the population of Nice, where two trapping locations were distinguishable (Figure 1). The first patch contained a group of 21 trees and the second patch 15 trees. Trees were planted a few meters from one another within patches, and the two patches were about 200 m away. Six sampled trees further apart were not included in this scheme (black dots on Figure 1).

We calculated the relatedness between individuals of each sex within and between patches using COANCESTRY software (Wang, 2011). Sex-biased dispersal should result in higher coefficients of relatedness within patches than between patches for the sex dispersing at smaller distances (Prugnolle and de Meeus, 2002). Coefficients of relatedness inter-patch and intra-patch were compared via a Kruskal-Wallis test (with patch as an explanatory factor) for each sex separately. For the previous significant test, post-hoc (with Bonferroni corrections) Mann-Whitney's *U*-tests between the three modalities (*i.e.* relatedness within patch 1, relatedness within patch 2, and relatedness between patches) were performed.

Effect of male density and sib frequency on sib mating avoidance

We aimed at untangling the effects of male density and the frequency of encounters with related males on female mate choice. We thus designed a factorial experiment with two

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densities of males (high density, noted *D*, with nine males, and low density, noted *d*, with three males) and two frequencies of related males (high frequency, noted *F*, with 2/3 of brothers, and low frequency, noted *f*, with 1/3 of brothers). Males were marked according to kinship (brother *vs* unrelated) with a drop of paint applied on their thorax 24h before being tested. The two colours used did not modify behaviours (data not shown, and(Desouhant et al., 2003) but were nonetheless alternated randomly across the different trials. Each trial took place as follows: a virgin female, kept in an individual vial from emergence, was released in a mesh cage (30 × 30 × 30cm) containing the males at 25°C, 65% RH and with a constant luminosity between 10 am and 4 pm. Courtship and mating behaviours were monitored for 30min or until a mating had occurred. We recorded mating latency, copulation duration, status (colour) of the successful male, number and timing of male rejections by females and status (colour) of rejected males. Twenty trials were performed per factor level, excepted for d-F (18 replicates). We analysed the proportion of sib mating with the GEE-GLM approach (Generalized Estimating Equation – Generalized Linear Model; (Liang and Zeger, 1986) implemented with a binomial distribution and a logit link function, an exchangeable correlation structure and a fixed scale. The model was fitted with the GEEPACK package in R software with families as clusters (Højsgaard et al., 2006). We also tested whether the latency before copulation was impacted by the density of males, the proportion of related males and the number and timing of rejected males (either related or unrelated) with a Cox model with mixed effects, with the families as random effect (COXME package in R software, (Therneau, 2015).

Results

Genotyping

A total of 241 wasps were captured in Valence and in Nice. In Valence, 78 wild females were captured (Table 1), among which 44 produced a total of 86 daughters (Table 1; 5.1 ± 0.4 offspring including 2.0 ± 0.2 daughters per female). In Nice, 86 males and 77 females were captured, among which 57 produced 140 daughters (Table 1; 4.8 ± 0.3 offspring including 2.5 ± 0.1 daughters per female). We genotyped 467 of these individuals at 18 microsatellite markers. We obtained the genotype at all 18 microsatellites for 402 individuals; for 50 individuals, 15 to 17 markers were amplified, for 11 individuals, 1 to 10 markers were amplified, and for four individuals, we obtained no amplification (Table 1). Only individuals with successful amplification at 15 loci or more were included in the dataset, which represented 452 individuals (96.8%).

No linkage disequilibrium was detected and null allele frequency was always lower than 6.5% in both populations. We found no genetic differentiation between the two populations (F_{st} = 0.01). We nonetheless treated the two populations separately because the probability that a male captured in one population had inseminated a female captured in the other is negligible. Effective population sizes was estimated at 79 for Nice (IC 95% [44; 82]) and 51 for Valence (IC 95% [26; 56]). These values are low and not significantly different.

Table 1 – Number of adult *Venturia canescens* captured in the wild populations of Nice and Valence. Pop = population; Number of mothers = number of females captured in the field and which have produced at least one daughter; F = female, M = male.

Pop	Sex	Wild individuals	Wild individuals successfully genotyped	Number of mothers	Total number of daughters	Daughters successfully genotyped
Nice	F	77	77 (100%)	54	140	134 (96%)
	M	86	79 (92%)	-	-	-
Valence	F	78	77 (99%)	35	86	85 (99%)

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Sib mating avoidance in the field? Relatedness of actual versus potential mates

In Nice, the mean coefficient of relatedness between wild males and females was $r = 0.047 \pm 0.001$. We estimated that 11% ± 0.79% (IC 95%) of the relatedness coefficients between these individuals were higher than the threshold $r_{crit} = 0.16$ (671 comparisons over 6083, from 79 males × 77 females); the corresponding pairs were considered as genetically related. The average probability that a female encountered a related male in the field the day of its capture was 12.0% ± 4.9 (Fig. 2).

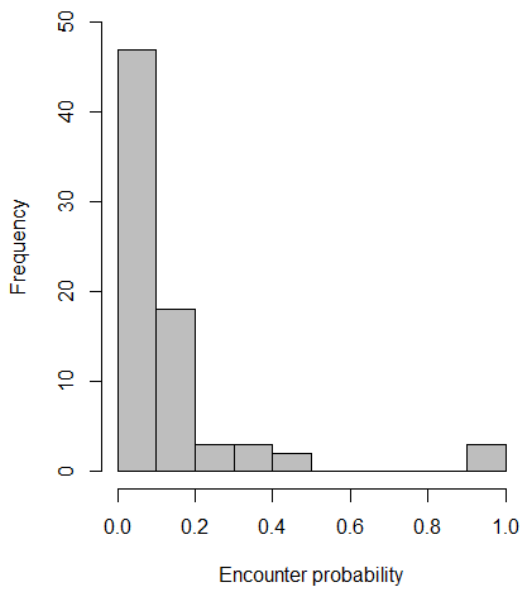

Figure 2 – Distribution of the probability that a female *Venturia canescens* encountered a genetically related male during the day of capture in the Nice population. For each female caught, we computed its relatedness with all males captured the same day. We then counted the number of relationships with $r \geq 0.16$ and we divided this number by the total number of relatedness computed (*i.e.* the number of males captured the same day).

Thirty-five and 54 wild females from Valence and Nice, respectively, produced at least one female offspring, allowing us to infer the genotype of their male mate (Table 1). The mean relatedness between these inferred pairs was 0.050 ± 0.009 in Nice and 0.044 ± 0.013 in Valence. Assuming $r_{crit} = 0.16$, we found six and four genetically related pairs in Nice and Valence, respectively, that is, 11.1% ± 8.4% and 11.4% ± 10.5% of all pairs. For the population of Nice, this observed number of related pairs did not differ from the number expected under the assumption of random mating (1000 permutations; mean number of related pairs expected under random mating assumption = 5.264; number observed = 6, $p = 0.822$; Fig. 3).

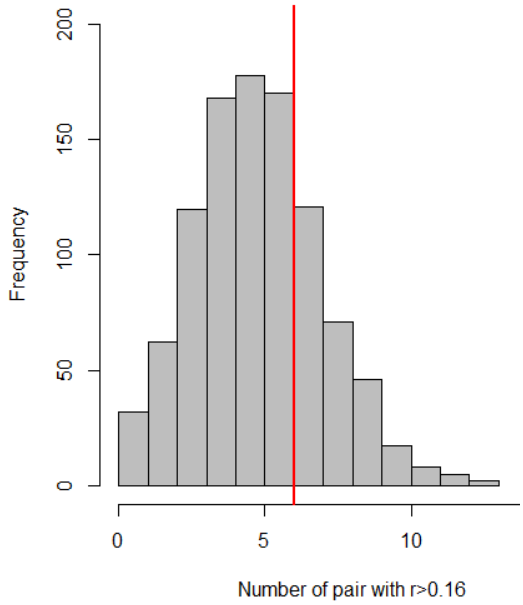

Figure 3 – Theoretical distribution of the number of crosses between genetically related male and female *Venturia canescens* under the assumption of random mating. The theoretical distribution was built using random sampling of a mate for each of the 51 females from the Nice population, estimating r for each pair and counting those with $r > 0.16$. Sampling was reiterated 1000 times. The red line represents the observed number of pairs with a $r > 0.16$.

Proportion of diploid males and number of CSD alleles

In species with sl-CSD, diploid males result from matched matings (parents sharing a common allele at the *csd* locus), and the probability of matched mating in a panmictic population is $2/k$, where k is the effective number of *csd* alleles. Hence, the number of alleles at the *csd* locus is inversely proportional to the frequency of diploid males (Cook, 1993; Cook and Crozier, 1995). We estimated the number of *csd* alleles in the population of Nice, where random mating is suggested by the

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previous result. Six males were found diploid among 79 males captured (7.6% ± 5.8%, IC 95%) yielding an estimation of 13.8 alleles at the *csd* locus (83 diploid individuals/6 diploid males) and a probability $p = 0.14$ of matched matings.

Sex-biased dispersal in the field

We captured 50 females and 47 males in patch 1, 18 females and 22 males in patch 2. Genetic relatedness was estimated among females and among males within patches (referred as 1-1 for intra Patch 1 and 2-2 for intra Patch 2) as well as between patches (referred as 1-2) (Table 2). Mean relatedness differed significantly within and between patches for females (Kruskal-Wallis test: $\chi^2 = 14.88$, $df = 2$, $p < 1.10^{-4}$) but not for males (Kruskal- Wallis test: $\chi^2 = 1.72$, $df = 2$, $p = 0.424$).

For females, relatedness was similar within each of two patches (1-1 and 2-2; Mann-Whitney test: $U = 80550$, $p = 0.520$; Table 2) and higher than between patches (1-1 / 1-2 comparison, Mann-Whitney test: $U = 56724$, $p < 4.10^{-4}$; 2-2 / 1-2 comparison, Mann-Whitney test: $U = 65356$, $p = 0.012$). Therefore, sex-biased dispersal occurs in *V. canescens*, with males dispersing more than females.

Effect of male density and sib frequency on sib mating avoidance

Mating success was higher at high male density, with 40 matings / 49 trials (81.6%) in high density conditions (*D*) versus 38 matings / 86 trials (44.1%) in low density conditions (*d*) (Table 3; proportion test: $\chi^2 = 16.44$, $df = 1$, $p = 5.02 .10^{-5}$). Mate choice did not depart from expected proportions of sib mating under random mating (GEE-GLM model, *p*-value of the Intercept = 0.3). This was confirmed by a positive relationship between the proportion of related males and the probability of sib mating (analysis of the Wald statistic table with an ANOVA, $\chi^2 = 15.36$, $df = 1$, $p = 8.9 .10^{-5}$). Male density had no effect on mate choice ($\chi^2 = 0.92$, $df = 1$, $p = 0.34$) and there was no interaction between male density and the proportion of related males ($\chi^2 = 0$, $df = 1$, $p = 1$). Time before copulation decreased with increasing male density and increasing number of sib rejections (Cox-hazard model with mixed effect, $p = 3.17.10^{-7}$ and $p = 1.36.10^{-3}$ respectively, Table 4), but was affected by neither the proportion of related males, nor the number of rejections of unrelated males ($p = 0.19$ and $p = 0.16$ respectively, Table 4).

Table 2 – Mean relatedness *r* (± SE) among females and among males of *Venturia canescens*, either within patches (1-1 or 2-2) or between patches (1-2) of host plants (carob trees) in the population of Nice. F = female, M = male.

Sex	Patch	Mean *r* (± SE)
F	1-1	0.065 ± 0.002
	2-2	0.060 ± 0.006
	1-2	0.050 ± 0.002
M	1-1	0.042 ± 0.002
	2-2	0.048 ± 0.006
	1-2	0.043 ± 0.003

Discussion

Sib mating avoidance in *Venturia canescens* had been evidenced from behavioural experiments in laboratory conditions (Metzger et al., 2010a). In contrast, the genetic approach developed in this study shows that in field populations, the observed frequency of matings between genetically related males and females is similar to the probability of sib mating expected under an assumption of completely random encounters. Our study thus suggests that sib mating tolerance occurs in the wild. A follow-up laboratory bioassay allowing mate choice further demonstrate that the density of males and the proportion of related individuals do not change the pattern of inbreeding avoidance: females do not use relatedness as a cue to choose their mates. This latter experiment nonetheless confirm that females can discriminate sibs from non-sibs and shows that

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the latency before copulation decreases after the rejection of a sib. Besides, our field data suggest sex-biased dispersal, although without yielding passive sib mating avoidance. In the remaining of the discussion, we propose an evolutionary scenario to explain the apparent discrepancy between patterns observed in field and in the laboratory.

Table 3 - Effect of male density and sib frequency on sib mating avoidance in *Venturia canescens*. Two densities of males (*D*: high density = 9 males, and *d*: low density = 3 males) and two frequencies of brothers (*F*: high frequency = 2/3 brothers and *f*: low frequency = 1/3 brothers) were manipulated in a cage experiment. Here, we present the number of successful matings for each modality (20, except for the *dF* modality where only 18 were reached).

	Mating	D	d
F	Brother	12	12
	Unrelated	8	6
	Successful	20/23 trials	18/44 trials
f	Brother	6	6
	Unrelated	14	14
	Successful	20/26 trials	20/42 trials

Mating rates between relatives, be they inferred from genetic analyses or from a simulation model assuming random encounter between males and females, were both equal to 11%. Consistently, in laboratory cages, the proportion of sib mating perfectly matched the proportion of sib present. Both results are congruent with an absence of sib mating avoidance in *Venturia canescens*, and questions past evidence of kin recognition in the contexts of both host parasitization (Bernstein and Driessen, 1996; Marris et al., 1996) and mate choice (Metzger et al., 2010a; Chuine et al., 2015). In their experiments, (Metzger et al., 2010a) showed with no-choice tests that a female mated twice more often with unrelated males than with their brothers, and were therefore capable of sib mating avoidance. Sib-mates were also discriminated against in the presence of odours from brothers, suggesting that mate-choice was underlined by volatile cues ((Bernstein and Driessen, 1996; Marris et al., 1996; Metzger et al., 2010a; Chuine et al., 2015). We confirmed this ability of kin recognition in our choice test, the rejection of brothers (but not that of unrelated males) decreasing the female copulation latency.

We propose several hypotheses to explain these differences in results. First, we could face the ghost of a past selection for sib mating avoidance. If the cost of inbreeding was higher in the evolutionary history of *V. canescens*, behaviours allowing kin recognition and reducing the risk of inbreeding could have been selected for. We found male-biased dispersal at a local scale (Table 2), which is quite rare in non-social insects (Lagisz et al., 2010; Bennett et al., 2013; Downey et al., 2015). This mechanism of sex-biased dispersal is sometimes responsible for inbreeding avoidance (Gandon, 1999; Roze and Rousset, 2005). For instance, in the mountain roe deer *Capreolus capreolus*, sib avoidance occurs during the mating season (summer). This genetic pattern is probably underlined by short-range breeding excursions by females, outside their usual home range, a behaviour which can decrease the probability of mating with a relative (Biosa et al., 2015). Although sex-biased dispersal seems to have no influence on sib mating in our population, it could decrease the selective pressure acting on sib mating avoidance by reducing the sib encounter rate. We indeed found that the distribution of sib-encounter probability is skewed towards zero, leading to more than half of the females having no chance to encounter a related male on a particular day, possibly because of the low number of individuals in the field or as a result of sex-biased dispersal (Fig. 2). The average probability for a female to encounter a relative is nonetheless in the same range as actual sib mating (12%; Fig. 2). Kin recognition mechanisms could then have persisted, but not sib mating avoidance.

The second hypothesis is that female choosiness in *V. canescens* is a density-dependent mechanism, as shown in other species (Kokko and Rankin, 2006; Duthie and Reid, 2016). Sib mating avoidance would arise

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in dense or aggregated populations with high mate encounter rates, but not in scarce populations where the cost of discrimination – remaining virgin – is higher. This was shown in a small isolated population of moose, with females accepting higher levels of inbreeding in years where males are less available (Herfindal et al., 2014). A similar reasoning may also hold for highly inbred populations, where sib mating avoidance would result in a high probability of not mating at all, a penalty that could exceed inbreeding depression (Kokko and Ots, 2006). In *V. canescens*, density affected mating success but not sib mating probability. Besides, the proportions of related males that we tested in the laboratory were respectively twice and four times higher than those found in the field, and still, no sib mating avoidance was observed. We therefore conclude that *V. canescens* does not avoid sib mating, whatever the density of males and the level of inbreeding.

Table 4 – Effect of male density, sib frequency and the number of male rejections on time before mating in *Venturia canescens*. We analysed the deviance table of the Mixed-effect Cox model with the female families as random effect.

	Log likelihood	χ^2	*df*	*p*-value
NULL	-351.51			
Male density	-338.44	26.14	1	$3.17 .10^{-7}$
Sib frequency	-337.57	1.74	1	0.19
Rejection of sib	-332.45	10.25	1	$1.36 .10^{-3}$
Rejection of unrelated	-331.46	1.97	1	0.16
Density × sib frequency	-331.46	0.01	1	0.92

The third hypothesis is that the absence of sib mating avoidance despite kin recognition could arise if female discrimination against related males decays over time. In a number of species, females adapt future mate choice according to the genotype of their first mate (lizards: (Laloi et al., 2011; Breedveld and Fitze, 2015), beetles: (Dowling et al., 2007), Drosophila: (Chapman and Partridge, 1998)). However, this hypothesis does not hold for *V. canescens* because females are monoandrous (Metzger et al., 2010b). Nonetheless, encountering a succession of low-quality (*i.e.* related) males could still affect female choice. We have shown in the lab experiment that even if females did not avoid sib mating, their latency before copulation decreased significantly after having rejected a brother. This result confirms that *V. canescens* is capable of discriminating relatives in the context of mating (Metzger et al., 2010a; Chuine, 2014; Chuine et al., 2015) and suggests that the presence of relatives affects mating by decreasing the latency before copulation.

An alternative argument to explain the observed lack of evidence for kin discrimination in our field study is the misclassification of cousins or more distant individuals as full sibs. The reasoning is that when genetic relatedness decreases to that of cousins ($r \leq 0.1875$) sib mating avoidance in *V. canescens* declines steeply ((Chuine, 2014). Hence, misclassifying cousins as brothers should yield an absence of mating avoidance, with no surprise. This is nonetheless unlikely. First, we classified pairs as sibs versus non-sibs based on a threshold $r_{crit} = 0.16$ that proved to minimize the probability of misclassification (about 10% of errors). Second, increasing this threshold to further restrict the definition of kinship did not change our conclusions, that is, the observed number of related pairs was always similar to that expected under random mating ($r_{crit} = 0.2$: 1000 permutations, 2 observed related pairs, 1.88 expected related pairs, $p = 1$; $r_{crit} = 0.25$: 1000 permutations, 0 observed related pairs, 0.999 expected related pairs, $p = 0.637$). Hence, the hypothesis that poor kinship assignation has yielded spurious results falls short of argument, and we conclude that the lack of

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evidence for sib mating avoidance truly reflects a lack of sib mating avoidance.

Tolerance to sib mating, as observed in *Venturia canescens*, is often explained by low inbreeding depression or high cost of outbreeding (Kokko and Ots, 2006; Puurtinen, 2011). In *V. canescens*, inbreeding has only a moderate negative impact on egg load and hatching rate, and no effect on other fitness components such as mating probability or body size (Vayssade et al., 2014). Despite common beliefs, the genetic load resulting from sl-CSD might not be dramatic either. In the population of Nice, we estimated 13.8 alleles at the *csd* locus, which is in the range of what was previously found in Hymenoptera (8-86 alleles, mostly between 9 to 20; (Cook and Crozier, 1995). Under random mating, this yields a probability of matched-mating of 14% (2/13.8). In case of brother sister-mating, the probability of matched mating increase to 50%. Assuming that females fertilize half of their eggs so that matched mating results in 25% diploid males, and that diploid males are sterile (Fauvergue et al. 2015), the expected reduction in the number of fertile offspring is 3.5% (0.14 x 0.25) under random mating. If sib mating was perfectly avoided, this expected reduction would be 2.3% (0.09 x 0.25, see SEM-E for the calculation). Sib mating avoidance would thus result in about 1% more fertile offspring in the populations we studied, a fitness gain that may appear insignificant. Hence, the relatively low probability of matched mating and the consequent benign inbreeding load may be insufficient to select for sib mating avoidance.

V. canescens could satisfy the hypothesis of inbreeding tolerance (Waser et al., 1986; Kokko and Ots, 2006). Tolerance to sib mating is found in other taxa, such as mammals or birds (Szulkin et al., 2009; Nichols et al., 2014; Reynolds et al., 2014), despite evidence for costs of inbreeding (Billing et al., 2012; Olson et al., 2012; Reid et al., 2015). However, we also found a low effective size in both sample sites (Ne = 51 in Valence, Ne = 79 in Nice), in the same range as threated bumblebees populations (Ellis et al., 2006) and some endangered populations of Hine's emerald dragonfly (Monroe and Britten, 2015). Therefore, *V. canescens* can be prone to loss of genetic diversity, which can be amplified by inbreeding tolerance. However, the *csd* alleles are under strong balancing selection, and thus could overcome the genetic impoverishment as in populations of Asiatic honeybee (Gloag et al., 2016).

At last, the discrepancies between sib mating avoidance in the lab and inbreeding tolerance in the field could come from the fact that kin-recognition mechanisms could have evolved for other purposes than sib mating avoidance. Evidence for active kin recognition is indeed pervasive in other ecological contexts such as territory defence, reciprocal altruism, or dominance hierarchies and so on (Mateo, 2004). In *Venturia* canescens, the evolution of kin-recognition in the superparasitism avoidance context could explain the maintenance of recognition without sib mating avoidance. Indeed, females are able to discriminate the self, their relatives and their conspecifics when laying their eggs, by avoiding already parasitized hosts (superparasitism avoidance). As *V.canescens* is a solitary species, only one offspring at most will emerge from a host and therefore superparasitism has a high cost for the female fitness. Moreover, the intensity of superparasitism avoidance increases with their relatedness to the first laying female (Hubbard et al., 1987; Marris et al., 1996; Amat et al., 2009). Under the hypothesis of a primary evolution of kin recognition in the parasitism context and a secondary use in mate choice, the molecular pathways of sib recognition in the two ecological contexts should therefore be close. Previous work has shown that kin recognition during mating was probably mediated by semiochemicals from the insect cuticle in this species (cuticular hydrocarbons, (Metzger et al., 2010a; Chuine, 2014). On the other hand, the discrimination of superparasitized larvae is driven by the chemical volatiles (hydrocarbons) that are left by the female during oviposition (Fisher, 1961; Harrison et al., 1985). The similarities between both chemical information enable us to question the existence of a unique pathway for the kin recognition in both contexts and therefore reconcile the laboratory results with our field study. The

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superparasitism avoidance could be a strong selective pressure linked to the maintenance of kin recognition, as the costs of superparasitism are positively correlated with the relatedness between the competing females. Further experiments are needed to appraise the hypothesis that the sib mating avoidance previously observed in the lab could be a by-product of this recognition.

Authors' contributions

M.C, I.A., X.F., L.M. and E.D. designed the experiment and M.C., S.S and A.A collected the data. M.C., I.A., L.M. and E.D. designed and interpreted the analyses. M.C. conducted the analyses and assembled a first draft. M.C., I.A., X.F., L.M. and E.D. all contributed to revisions.

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Data accessibility

The data set used for the main analyses, the scripts to recreate the results and the figures are available from the Dryad Digital Repository http://dx.doi.org/

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