Insects and incest: sib-mating tolerance in natural populations of a parasitoid wasp

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Abstract

1. Sib-mating avoidance is a pervasive behaviour, which likely evolves in species that are 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 lab experiment and 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 as a consequence, sib-mating is partially avoided.

3. Our study consisted of a mate choice experiment in laboratory cages and a field study based on the genotyping of 86 wild-caught males, 155 wild-caught females and their 226 daughters at eighteen microsatellite loci. With these field data, we reconstructed the genotype of each female's mate and estimated the relatedness of each mating pair.

4. The mate choice experiment confirmed that kin discrimination occurs in this species. Time to mating depended on the frequency of female encounters with related and

unrelated males. However, contrary to previously published results, no sib-mating avoidance was detected during these experiments. In the field, we found that the effective rate of sib-mating did not differ from the probability that sibs encounter at random, which corroborates the absence of sib-mating avoidance. We also detected a weak but significant male bias in dispersal, a pattern which could reduce encounters between sibs.

5. These results suggest that despite kin discrimination, *Venturia canescens* tolerates sib-mating in the field. The weak male-biased dispersal cannot explain entirely this pattern. The observed sib-mating tolerance raises the question as to why kin discrimination is maintained in this species. It also calls into question common beliefs on inbreeding depression in species with single-locus complementary sex determination.

Key-words: Field study**,** Inbreeding tolerance, Kin recognition, Parasitic wasp, single-locus Complementary Sex Determination, sl-CSD.

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 referred to as inbreeding depression (Pusey and Wolf, 1996; Keller and Waller, 2002; Angeloni et al., 2011). Inbreeding depression is caused by the expression of deleterious recessive alleles at the homozygous state or by a loss of heterosis in inbred crosses (Charlesworth and Charlesworth, 1987; Pusey and Wolf, 1996). Selection on behaviours underlying inbreeding avoidance should thus depend on inbreeding load, which balances the advantages of inbreeding avoidance with the costs of implementing adapted physiological or behavioural responses. Selection may also depend on the benefits of inbreeding in terms of inclusive fitness, as inbreeding results in an increased representation of genes identical by descent in the offspring (Kokko and Ots, 2006; Puurtinen, 2011; Duthie and Reid, 2016).

Responses to inbreeding risk 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* ; in this species, inbreeding does not lower the survival and growth rate of the youngs, and related pairs invest more in parental care and cooperate better (Thünken et al., 2007). In rodents such as *Marmota flavipentris*, inbreeding preference was shown despite the lower survival of inbred progeny. This preference can be explained by the large variation of male reproductive success and the composition of social groups, where males mating with related females do not suffer decreased annual reproductive success compared to the majority of males, that achieve little or no reproductive success (Olson et al., 2012). In contrast, ring-tailed lemurs, or mole rats living in socially structured groups, avoid inbreeding. In mole rats, avoidance leads to a high reproductive skew in males, with a subset of males that never access to reproduction (Cooney and Bennett, 2000). In ring-tailed lemurs, inbreeding avoidance allows females to save energy (pregnancy and lactation) that would be lost for the production of inbred offspring suffering from depressed immune functions and a reduced life expectancy (Boulet et al., 2009; Charpentier et al., 2008). Hence, quantifying the costs and benefits of inbreeding avoidance and the balance between them is necessary to decipher the selective pressures acting on inbreeding avoidance, including the consequences of inbreeding, and in turn, to understand the evolution of this behaviour.

Additionally, the balance between benefits and costs associated with inbreeding avoidance depends on environmental factors, such as population density, spatial or social population structure. A low 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 selectivity 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). In cooperative breeding species, inbreeding avoidance strategies can lead to the suppression of reproduction outside dominant pairs due to the lack of opportunity of outbred matings (as in the Damaraland mole-rats *Fukomys damarensis*; (Cooney and Bennett, 2000). Therefore, the reproductively supressed subordinates that are related to the dominant pair should increase their involvement in help to increase their own inclusive fitness and maintain the social structure of the group (Nichols, 2017). This interplay between environment and inbreeding avoidance patterns requires field data to be able to quantify cost and benefits of each strategy.

Various behavioural and physiological strategies to avoid inbreeding risks have been selected for 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 probably the most pervasive behaviour for sib-mating avoidance (Pusey and Wolf, 1996) and relies mostly on sib recognition mediated by chemical communication (Howard and Blomquist, 2005; Johansson and Jones, 2007; Charpentier et al., 2008a; Bonadonna and Sanz-Aguilar, 2012). It is nonetheless important to note that kin recognition mechanisms can arise from selective forces other than inbreeding avoidance, such as nest-mate recognition, parental care, territory defence, dominance hierarchies to name a few (Mateo, 2004).

Assessing inbreeding avoidance patterns, 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 and 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 not surprising that inbreeding avoidance patterns have been rarely documented in insects in the wild (but see Robinson et al., 2012 for a field assessment of optimal inbreeding in *Drosophila melanogaster*). Laboratory studies nonetheless suggest various strategies of inbreeding avoidance in different insect genus, from active choices such as precopulatory avoidance of related males, which represent low quality partners with a high genetic load, in the butterfly *Bicyclus anynana* (Fischer et al., 2015) or postcopulatory choice with the preferential use of the sperm stored from unrelated males in *Gryllus bimaculatus* (Bretman et al., 2004, 2009). In a social species of termite, *Neotermes chilensis*, the dispersal of individuals founding new colonies is the main mechanism to avoid inbreeding risks (Aguilera-Olivares et al., 2015). Absence of inbreeding avoidance has also been evidenced in insects such as parasitoid wasps (Bourdais and Hance, 2009) or *Drosophila melanogaster* (Tan et al., 2012). The diversity of strategies unveiled in the laboratory renders more striking our lack of knowledge on insect inbreeding avoidance patterns *in natura*, and calls for field studies.

Here, we take up this challenge by studying, both in the laboratory and 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; Asplen et al., 2009; Schmieder et al., 2012). In haplodiploid species, 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, if they mate, half of their diploid offspring will develop 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 deleterious alleles at the sex locus but a strong

121 heterozygous advantage (e.g., Charlesworth and Willis, 2009). The null fitness of diploid males should
122 favour sib-mating avoidance.

123 The parasitoid wasp *Venturia canescens* Gravenhost (Hymenoptera: Ichneumonidae) has a
124 single locus complementary sex determination (first demonstrated by Beukeboom, 2001) and
125 inbreeding reduces the fitness of both males and females via the production of sterile diploid offspring
126 and a negative impact on egg load and hatching rate (Vayssade et al., 2014; Chuine et al., 2015).
127 Consistently, in no-choice bioassays, mating success decreases with increasing genetic relatedness
128 between mates (Metzger et al., 2010a; Chuine, 2014). Circumstantial evidence suggests that females
129 are the choosy sex, which makes sense in a species with a monoandrous/polygenous mating system
130 (Metzger et al., 2010a). However, when confronting females to choice between a brother and an
131 unrelated male, inbreeding avoidance vanishes, likely because the mixture of volatiles in small test
132 tubes confounds females (Metzger et al., 2010a).

133 In this study, we address the question of inbreeding avoidance by combining field and
134 laboratory approaches. We first tested the effects of male density and average relatedness on
135 inbreeding avoidance with more realistic conditions than before (Metzger et al., 2010a). We expected
136 sib-mating avoidance to decrease with decreasing density and increasing relatedness. We further
137 implemented a population genetic approach based on microsatellite genotyping in order to assess
138 inbreeding avoidance and underlying behaviours in field populations of *V. canescens*. For this, we
139 sampled two natural populations of *V. canescens* and compared the observed rate of sib-mating to the
140 probability of random encounter among genetically-related individuals. Under the hypothesis of sib-
141 mating avoidance, we expected the rate of sib-mating to be smaller than the probability that relatives
142 encounter at random. Besides, we used the field data to test whether sex-biased dispersal limits
143 encounters among sibs in the field. We show that sib-mating tolerance occurs in the wild, and propose
144 an evolutionary scenario to explain why this result contrasts with earlier findings of inbreeding

avoidance in this species as well as prior expectations about inbreeding depression in species with sl-CSD (van Wilgenburg et al., 2006).

Materials and Methods

Biological model

Venturia canescens is a monoandrous solitary endoparasitoid found in most circum-Mediterranean areas (Salt, 1976). Females lay eggs in a large range of hosts found on various fruits (Salt, 1976; Driessen and Bernstein, 1999). Knowledge of the mating system of *V. canescens* under natural conditions is limited but can partly be inferred from lab results. Females do not discriminate between host patches with more than four host larvae, suggesting that, in the field, they rarely encounter aggregated host (Driessen and Bernstein, 1999). Therefore, it is unlikely that this species experiences local mate competition. Males search for females via a synergistic combination of volatile sex pheromones emitted by females and kairomones from hosts (Metzger et al., 2010b). Both immature development and adult life last around three weeks (at 25°C), and females lay eggs during most of their adult life (Metzger et al., 2008). This leads to overlapping generations. Depending on studies, sex-ratio is considered balanced (Beukeboom, 2001) or weakly biased toward females (Metzger et al., 2008). Balanced sex ratio is congruent with the absence of aggregation (Driessen and Bernstein, 1999), the absence of local mate competition, and mate-finding via volatile cues. The proportion of diploid males corresponds to that expected under the hypothesis of a balanced sex ratio (Fauvergue et al., 2015). Field and laboratory studies showed that adult females are good dispersers, with a flight velocity estimated at 0.2 $m.s^{-1}$ (Schneider et al., 2002; Desouhant et al 2003; Amat et al., 2012).

Insect sampling in the field

Adults *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-baited traps for the capture of live females, and yellow sticky traps for males (which were generally found dead). Female traps constituted of an open cup containing host larvae, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), along with used rearing medium (semolina). Saliva secreted by host larvae when feeding serves as a kairomone for female *V. canescens* (Corbet, 1971). Traps were hung in trees (Metzger et al., 2008) and visited every 20 minutes to capture attracted females. For males, 125 mm × 200 mm yellow sticky cards were baited with extracts from females and hosts, a method that has already proved successful (Collet et al., 2016). For each trap, a vial containing the extracts was attached at the centre of the card. 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 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 a climatic chamber under constant conditions (24±1°C, 60±10% RH, LD 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 we could not control for the age and previous egg-laying experience of captured females, the number of offspring was highly variable (1-10 daughters and 1-7 sons). As sexual (arrhenotokous) and asexual (thelytokous) 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, and thelytokous individuals were discarded. Mothers and their offspring were then preserved individually in 96% ethanol.

For laboratory experiments, we used a standardized laboratory rearing. The wasp culture had been established with about 60 females collected in the field near Valence (same location as the field capture, N 44°58'21" E 4°55'39", INRA, Gotheron), southern France, on several occasions during the summer 2015. *Ephestia kuehniella* was reared in the laboratory on organic wheat semolina medium for three weeks before being exposed to parasitoids (eggs were obtained from the Biotop rearing facility located in Livron sur Drôme, France). Parasitoid development took place in a controlled environment (24±1°C; 60±5% RH; LD regime 12 h:12 h).

Experiments on female mate choice required related and unrelated males. We thus initiated families by collecting females randomly from the mass rearing (from different rearing boxes), and maintaining them individually in a little circular box (Ø: 8 cm, height: 3 cm) with meshed lid with two males and about 30 three-weeks host larvae on 1 cm of organic wheat semolina. The wasps were fed *ad libitum* with water-diluted honey (50-50) and allowed to lay eggs during their whole life. About three weeks after, the parents were removed from the box if still alive, and their offspring were collected at emergence, before reproduction. Females (the daughters) were kept individually in plastic vials with a drop of water-diluted honey, and males were kept with their brothers and fed similarly. All individuals used in the experiments were two-days old.

Effect of male density and sib frequency on sib-mating avoidance in the laboratory

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 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 do not modify behaviours (data not shown, and Desouhant et al., 2003) but were nonetheless alternated randomly across trials. Each trial took place as follows: a virgin female was released in a mesh cage (30 × 30 × 30cm) containing

218 the males at 25°C, 65% RH and with a constant luminosity. Courtship and mating behaviours were
219 monitored for 30 min or until a mating had occur between 10 am and 4 pm. We recorded mating
220 latency, copulation duration, relatedness (colour) of the successful male, and courtship effort (*i.e.* the
221 time and the duration of each male courtship behaviour) (van Santen and Schneider 2002). For each
222 courtship, we identified the relatedness (colour) of the candidate. Therefore, all courtships were
223 counted independently of mating success. Twenty trials were performed per factor level, excepted for
224 *d-F* (18 replicates).

225 We analysed the proportion of sib-mating with a GEE-GLM (Generalized Estimating Equation –
226 Generalized Linear Model; Liang and Zeger, 1986, GEEPACK package in R software) implemented with a
227 binomial distribution of error, a logit link function, an exchangeable correlation structure, and a fixed
228 scale. The model was fitted with mate relatedness as a response variable (binomial: 0 if the female
229 mated with her brother, 1 if she mated with an unrelated male), density and frequency of brothers as
230 main effects and in interaction as explanatory variables, and families as a random factor (*i.e.* clusters,
231 Højsgaard et al., 2006). We added an offset term to take into account the theoretical mating
232 proportion with related males (*i.e.* 1/3 in low frequency *f* and 2/3 in high frequency *F*).

233 We also tested the effects of density and proportion of brothers on male courtship effort and latency
234 before copulation. For courtship effort, we implemented a GLM based on a binomial distribution and
235 a logit link function, with the percentage of courtships done by brothers as a response variable and the
236 density and proportion of brothers as explanatory variables. For latency before copulation, we
237 performed a Cox model with the same explanatory variables as fixed effects and family as a random
238 effect (COXME package, Therneau, 2015 in R software). We also included the number and timing of
239 courtships (from a related or unrelated male) as a time-dependent variable. If females assess their
240 relatedness with males during courtship, an increasing number of encounters with related males could
241 lead the female to decrease its selectivity and, as a consequence, the latency before mating. In the

242 other hand, an increasing number of encounters with unrelated males should not affect its selectivity
243 and therefore its latency to mate.

244 *Genotyping*

245 Genotyping was performed at 18 microsatellite loci developed by Collet et al. (2016) and
246 Mateo Leach et al. (2012) (See SEM-A for details on the genotyping and SEM-D for details on the
247 number of microsatellites used). We genotyped all field-captured males and females (hereafter
248 referred to as wild females) and up to three daughters per female (see SEM-B for a justification of the
249 number of offspring genotyped). When fewer than three daughters emerged (ca. 34%, in which 40%
250 produced only one daughter), all were genotyped.

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

261 *Inference of relatedness among mates*

262 - Male genotypes

263 We inferred the genotype of wild females' mates based on the genotypes of wild females and
264 that of their daughters. For this, we used the software COLONY, which reconstructs families based on
265 haplodiploid genotypes (Jones and Wang, 2010). When alternative genotypes were proposed for a

given male, we selected the one that was compatible with the genotype of the daughter and the mother. When several alternatives were possible (e.g. if mother and daughters were heterozygous with the same alleles at a given locus), it was impossible to assign the right allele to the father with certitude. In this case, we assigned the allele with the highest probability of occurrence (the probability is given by COLONY). Doing so, we successfully inferred the genotype of 51 mates for the 54 wild females captured (see Results and Table 1).

- Relatedness between mates

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 genetically 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 (see SEM-C for details on simulations). Simulations allowed to keep track of pedigrees, yielding a perfect knowledge of kinship for all potential mates, which we classified in three categories: full-sibs, half-sibs, or unrelated. Using microsatellite genotypes from the same simulated dataset, we estimated the relatedness coefficient (r) for all female-male pairs (TrioML coefficient, software COANCESTRY; Wang, 2011). We then used these estimated relatedness coefficients with different relatedness thresholds to assign each dyad to related vs unrelated categories (SEM-C). By comparing these posterior assignations to the pedigrees known a priori, we estimated the relatedness threshold that minimized the number of wrong assignations (*i.e.* unrelated pairs assigned related based on the threshold, or vice-versa) under random encounter hypothesis. 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.

291 We applied this r_{crit} = 0.16 threshold to field data. First, we estimated the number of wild
292 females that had been inseminated by a related male. Second, we generated a theoretical distribution
293 of the number of couples with a r ≥ 0.16 under the null hypothesis of random mating. For this, each of
294 the 51 wild females from Nice, for which a mate genotype had been successfully inferred, was paired
295 randomly with one of the 79 genotyped males, and we counted the number of pairs with r ≥ 0.16. This
296 calculation was repeated 1000 times to produce the null distribution. We finally compared the
297 observed number of related pairs with the null distribution via a permutation test implemented in the
298 function AS.RANDTEST from the ADE4 package in R software version 3.2.3 (Chessel et al., 2004; R Core
299 Team, 2015). We also estimated the probability that a female encountered a related male in the field
300 the day of her capture. Indeed, partners present in the same day in the same area have a high chance
301 to encounter each other and mate due to the high dispersal capacity of this species.

302 *Sex-biased dispersal in the field*

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

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

315 relatedness within patch 1, relatedness within patch 2, and relatedness between patches) were
316 performed.

317 **Results**

318 *Effect of male density and sib frequency on sib-mating avoidance in the laboratory*

319 Mating success was higher at high male density (*D;* 81.6%, 40 matings/49 trials) than at low
320 density (*d*; 44.1%; 38/86) (Table 3; proportion test: $\chi^2 = 16.44$, $df = 1$, $p = 5.02\ .10^{-5}$). The observed
321 proportion of sib-mating did not depart from that expected under random encounters (GEE-GLM
322 model, *p*-value of the intercept = 0.3). This was confirmed by a positive relationship between the
323 proportion of related males and the probability of sib-mating (Wald statistic table, $\chi^2 = 15.36$, $df = 1$,
324 $p = 8.9\ .10^{-5}$). Male density had no effect on mate choice, neither as a main effect ($\chi^2 = 0.92$, $df = 1$,
325 p = 0.34) nor in interaction with the proportion of related males ($\chi^2 = 0$, $df = 1$, $p = 1$). Males did not
326 differ in their courtship effort, as the proportion of courtships observed for unrelated males and
327 brothers did not depart from the expected values (GLM model, *p*-value of the Intercept = 0.16): for
328 example, in the *D-F* modality with 6 brothers and 3 unrelated males, we expected that 2/3 of the
329 courtships should be done by brothers (i.e. 13.3 on 20 matings) and observed 12 sib-mating. Time
330 before copulation decreased with increases in male density and increases in the number of sib
331 rejections (Table 4), but it was not affected by the proportion of related males or by the number of
332 rejections of unrelated males (Table 4).

333 *Genotyping*

334 A total of 241 wasps were captured in Valence and in Nice. In Valence, 78 wild females were
335 captured (Table 1) among which 35 produced a total of 86 daughters (Table 1; 5.1 ± 0.4 offspring
336 including 2.4 ± 0.2 daughters per female). In Nice, 86 males and 77 females were captured, among
337 which 54 produced 140 daughters (Table 1; 4.8 ± 0.3 offspring including 2.6 ± 0.1 daughters per
338 female). We genotyped 467 of these individuals at 18 microsatellite markers. We obtained the

genotype at all 18 microsatellite loci for 402 individuals, at 15-17 loci for 50 individuals, 1-10 loci for 11 individuals, and for four individuals, we obtained no amplification (Table 1). Only individuals with successful amplification at >14 loci 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 the two 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 300 km apart is negligible. Effective population sizes were estimated to be 79 for Nice (IC 95% [44; 82]) and 51 for Valence (IC 95% [26; 56]). These values are low and not significantly different.

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 ± 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 thus considered 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).

Thirty-five and 54 wild females from Valence and Nice, respectively, produced at least one daughter which allowed to infer the genotype of their 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 from random mating = 5.264; number observed = 6, p = 0.822; Fig. 3).

364 *Proportion of diploid males and number of CSD alleles*

365 Estimating inbreeding depression is required to understand the balance between costs and
366 benefits of inbreeding avoidance in a given species. Here, we assessed the cost of inbreeding by
367 estimating the number of diploid males in the population of Nice (no male was captured in Valence).
368 In species with sl-CSD, diploid males result from matched matings (parents sharing a common allele at
369 the *csd* locus), and the probability of matched mating in a panmictic population is $2/k$, where k is the
370 effective number of *csd* alleles. The number of alleles at the *csd* locus k could be estimated from the
371 proportion of diploid males among the males m and the proportion fertilization s (see SEM-E for the
372 calculation). As every evidence points to a balanced sex-ratio, we set s=0.5. We estimated the number
373 of *csd* alleles in the population of Nice, where random matings are suggested by the result on the
374 number of related pairs in the wild (see previous section). Six males were found diploids among the 79
375 males captured (m=7.6% ± 5.8%, IC 95%) yielding an estimation of 8.8 alleles at the *csd* and a
376 probability $2/k$ = 0.23 of matched matings.

377 *Sex-biased dispersal in the field*

378 During the field experiment, 50 females and 47 males were genotyped in patch 1, 18 females
379 and 22 males in patch 2. Genetic relatedness was estimated among females and among males within
380 patches (referred to as 1-1 for intra Patch 1 and 2-2 for intra Patch 2) as well as between patches
381 (referred to as 1-2) (Table 2). Mean relatedness differed within and between patches for females
382 (Kruskal-Wallis test: χ^2 = 14.88, df = 2, $p < 1.10^{-4}$) but not for males (Kruskal-Wallis test: χ^2 = 1.72, df =
383 2, p = 0.424). For females, relatedness was similar within each of two patches (1-1 and 2-2; Mann-
384 Whitney test: U = 80550, p = 0.520; Table 2) and higher than between patches (1-1 / 1-2 comparison,
385 Mann-Whitney test: U = 56724, $p < 4.10^{-4}$; 2-2 / 1-2 comparison, Mann-Whitney test: U = 65356, p =
386 0.012). Therefore, sex-biased dispersal occurs in *V. canescens*, with males dispersing more than
387 females.

388 **Discussion**

389 Sib-mating avoidance in *Venturia canescens* had been observed in no-choice behavioural
390 experiments conducted in laboratory conditions (Metzger et al., 2010a, Chuine, 2014). We show here
391 that when females can choose between related and unrelated males, they do mate with their sibs. The
392 density of males and the proportion of related individuals in the surroundings do not change the
393 pattern of inbreeding avoidance, suggesting that relatedness is not a cue for mate choice. Females
394 nonetheless appear to discriminate sibs from non-sibs, as shown by decreasing latency before
395 copulation after rejection of a sib. The sib-mating tolerance that we observed in the laboratory is
396 confirmed by our genetic approach of field populations. We showed that the observed frequency of
397 sib-mating corresponds to the probability of sib-mating expected from random encounters. Besides,
398 our field data suggest a slightly sex-biased dispersal, although without yielding passive sib-mating
399 avoidance. In the remainder of the discussion, we propose an evolutionary scenario to explain the
400 maintenance of kin recognition system and its apparent absence of use in the mate choice context, in
401 the lab or in the field.

402 In laboratory cages, the proportion of sib-mating perfectly matched the proportion of sibs
403 present. Consistently, mating rates between relatives, be they inferred from genetic analyses or from
404 a simulation model assuming random encounters, were both equal to 11%. These two results are
405 congruent with an absence of sib-mating avoidance in *V. canescens*, and questions past evidence of
406 kin recognition and avoidance in the contexts of both host parasitization (Bernstein and Driessen,
407 1996; Marris et al., 1996) and mate choice (Metzger et al., 2010a; Chuine et al., 2015). In particular,
408 no-choice bioassays had shown that females *V. canescens* mated twice more often with unrelated
409 males than with their brothers, and were therefore capable of sib-mating avoidance (Metzger et al.
410 2010a). In the presence of volatiles from brothers, females also discriminated against unrelated males,
411 suggesting that mate-choice could be underlined by volatile cues (Metzger et al., 2010a; Chuine et al.,
412 2015). Our laboratory cage experiment also suggests the ability of females to recognize kin and modify
413 a component of their mating behaviour (latency before copulation), but do not yield sib-mating

414 avoidance. We further discuss eight hypotheses to explain the differences between our findings and
415 those of Metzger et al., (2010a).

416 First, the observed lack of evidence for kin discrimination in our field study could result from a
417 misclassification of cousins or more distant individuals as full sibs. The reason is that when genetic
418 relatedness decreases to that of cousins ($r \leq 0.1875$) sib-mating avoidance in *V. canescens* declines
419 steeply (Chuine, 2014). Hence, misclassifying cousins as brothers would yield an absence of sib-mating
420 avoidance, with no surprise. This is nonetheless unlikely. We did classify pairs as sibs versus non-sibs
421 based on a threshold $r_{crit} = 0.16$ that proved to minimize the probability of misclassification (about 10%
422 of errors). Besides, increasing this threshold to further restrict the definition of kinship did not change
423 our conclusions, that is, the observed number of related pairs was always similar to that expected
424 under random matings ($r_{crit} = 0.2$: 1000 permutations, 2 observed related pairs, 1.88 expected related
425 pairs, $p = 1$; $r_{crit} = 0.25$: 1000 permutations, 0 observed related pairs, 0.999 expected related pairs, $p =$
426 0.637). Hence, the hypothesis that poor kinship assignation has yielded spurious results falls short of
427 argument, and we conclude that the lack of evidence for sib-mating avoidance truly reflects a lack of
428 sib-mating avoidance, called sib-mating tolerance.

429 The second hypothesis is that we could face the ghost of past selection for sib-mating
430 avoidance. If the cost of inbreeding was higher in the evolutionary history of *V. canescens*, behaviours
431 allowing kin recognition and reducing the risk of inbreeding could have been selected for.

432 A third hypothesis is that sex-biased dispersal shapes the pattern of inbreeding avoidance in
433 the field (Gandon, 1999; Roze and Rousset, 2005). In *V. canescens* we found a slight male-biased
434 dispersal at a local scale (Table 2). However, in the population of Nice, the relatedness values between
435 and within patches (between which dispersal occurs) in both sexes are low (between 0.042 and 0.065,
436 Table2). Therefore, if this male-biased dispersal affects the structure of this population and encounter
437 rates between relatives, it should be a weakly effect.

438 The fourth hypothesis is that female choosiness in *V. canescens* is density-dependent, as
439 shown in other species (Kokko and Rankin, 2006; Duthie and Reid, 2016). Sib-mating avoidance would
440 arise in dense or aggregated populations with high mate encounter rates, but not in scarce populations
441 where the cost of discrimination – remaining virgin – is higher. This was shown in a small isolated
442 population of moose, where females accepted mates with higher relatedness during years where
443 males were less available (Herfindal et al., 2014). A similar reasoning may also hold true for highly
444 inbred populations, where sib-mating avoidance would result in a high probability of not mating at all,
445 a penalty that could exceed inbreeding depression (Kokko and Ots, 2006). In *V. canescens*, population
446 density affects mating success but not sib-mating probability. Besides, the proportions of related males
447 that we tested in the laboratory were respectively twice and four times higher than those found in the
448 field, and still, no sib-mating avoidance was observed. In the field, the distribution of the sib-
449 encounters probability is skewed towards zero, leading to more than half of the *V. canescens* females
450 having no chance to encounter a related male on a particular day, possibly because of the low number
451 of individuals in the field (Fig. 2). The average probability for a female to encounter a relative is
452 nonetheless in the same range as actual sib-mating (12%; Fig. 2). Kin recognition mechanisms could
453 then have persisted, but not sib-mating avoidance. We therefore conclude that *V. canescens* does not
454 avoid sib-mating, whatever the density of males and the level of inbreeding.

455 A fifth hypothesis to explain the absence of sib-mating avoidance despite kin recognition ability
456 is that female discrimination against related males decays over time. Encountering a succession of low-
457 quality males (*i.e.* genetically-related in our study) could decrease female choosiness, as demonstrated
458 in a monoandrous wolf spider (Stoffer and Uetz, 2016). We have shown in the lab experiment that
459 even if females did not avoid sib-mating, their latency before copulation decreased significantly after
460 having rejected a brother. This result confirms that *V. canescens* is capable of discriminating relatives
461 in a context of mating (see also Metzger et al., 2010a; Chuine, 2014; Chuine et al., 2015) and suggests
462 that the presence of relatives could affect mating by decreasing the latency before copulation, as

463 expected in this hypothesis. We cannot therefore rule out this hypothesis but more experiments are
464 needed to firmly test the evolution of female choosiness over time.

465 The sixth hypothesis is that tolerance to sib-mating, as observed in *Venturia canescens*, is often
466 explained by low inbreeding depression or high cost of outbreeding (Kokko and Ots, 2006; Puurtinen,
467 2011). We found a low effective size in both sample sites (Ne = 51 in Valence, Ne = 79 in Nice), in the
468 same range as threatened bumblebees (Ellis et al., 2006) or Hine's emerald dragonflies (Monroe and
469 Britten, 2015). *V. canescens* could be prone to loss of genetic diversity, possibly amplified by inbreeding
470 tolerance, which would in turn increase inbreeding load. However, alleles at the *csd* locus, the major
471 mechanism of inbreeding depression in *V. canescens*, are under strong balancing selection, and are
472 thus expected to overcome genetic impoverishment, as observed in populations of Asiatic honeybee
473 (Gloag et al., 2016). Moreover, in *V. canescens*, inbreeding has only a moderate negative impact on
474 egg load and hatching rate, and no effect on other fitness components such as mating probability or
475 body size (Vayssade et al., 2014).

476 The seventh hypothesis is that despite common beliefs, the genetic load resulting from sl-CSD
477 might not be dramatic in *Venturia canescens*. In the population of Nice, we estimated 8.8 alleles at the
478 *csd* locus, which is in the range of what was previously found in other species of the order
479 Hymenoptera (8-86 alleles, mostly between 9 to 20; Cook and Crozier, 1995). Under random matings,
480 this yields a probability of matched-matings of 23% (2/8.8). In case of brother-sister mating, the
481 probability of matched matings increases to 50%. Assuming that females fertilize half of their eggs so
482 that matched matings results in 25% diploid males, and that diploid males are sterile (Fauvergue et al.
483 2015), the expected reduction in the number of fertile offspring under random mating would be 5.8%
484 (0.23 x 0.25). If sib-mating was perfectly avoided, the expected reduction in the number of fertile
485 offspring would be 4.8% (0.19 x 0.25, see SEM-E for the calculation). Sib-mating avoidance would thus
486 result in about 1% more fertile offspring in the populations we studied, a fitness gain that may appear
487 insignificant. Hence, the relatively low probability of matched matings and the consequent benign

488 inbreeding load may be insufficient to select for sib-mating avoidance. *V. canescens* could so satisfy
489 the hypothesis of inbreeding tolerance (Waser et al., 1986; Kokko and Ots, 2006).

490 At last, the eight hypothesis lean on the possibility that the discrepancies between sib-mating
491 avoidance evidenced in previous research and our finding of inbreeding tolerance could highlight the
492 evolution of kin-recognition mechanisms for other purposes than sib-mating avoidance. This would
493 have produced artefactual behaviours when observed in small arenas (Metzger et al., 2010a) but
494 vanishing in larger cages and in the field. Evidence for active kin recognition is pervasive in various
495 ecological contexts such as territory defence, reciprocal altruism, or dominance hierarchies (Mateo,
496 2004). In *V. canescens*, the evolution of kin recognition for the avoidance of superparasitism could
497 explain the maintenance of kin recognition without sib-mating avoidance. Indeed, females are able to
498 discriminate the self, their relatives and their conspecifics when laying eggs, and avoid already
499 parasitized hosts. As a single offspring can develop in a host, superparasitism has a high cost for female
500 fitness. Consistently, the intensity of superparasitism avoidance increases with genetic relatedness to
501 the first laying female (Hubbard et al., 1987; Marris et al., 1996; Amat et al., 2009). If kin recognition
502 evolved in this context, kin discrimination in a situation of mate choice could be a by-product of this
503 primary selection on kin recognition. We could therefore expect a relatively general molecular
504 pathway, which would have been expressed in a small-scale mating bioassay: kin recognition during
505 mating is probably mediated by semiochemicals from the insect cuticle (cuticular hydrocarbons,
506 Metzger et al., 2010a; Chuine, 2014) and discrimination against superparasitized larvae is driven by
507 weakly volatile hydrocarbons left by females during oviposition (Fisher, 1961; Harrison et al., 1985).
508 These mechanistic similarities point towards a unique pathway for kin recognition. Superparasitism
509 avoidance could act as a strong selective pressure for the maintenance of kin recognition, because the
510 cost of superparasitism is positively correlated with the relatedness among competing females.
511 Further experiments are needed to test the hypothesis that sib-mating avoidance observed in
512 laboratory conditions is a by-product of cognitive abilities which have evolved in a context of
513 competition.

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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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748 **Table 1 –** Number of adults *V. canescens* captured in the wild populations of Nice and Valence.
749 Pop = population; Number of mothers = number of wild females captured in the field and which have
750 produced at least one daughter; F = female, M = male.
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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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754 **Table 2 –** Mean relatedness *r* (± SE) among females and among males of *V. canescens*, either within
755 patches (1-1 or 2-2) or between patches (1-2) of host plants (carob trees) in the population of Nice.
756 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

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Table 3 - Effect of male density and sib frequency on sib-mating avoidance in *V. 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 factor level (20, except for the *d-F* modality where only 18 were reached).

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

766 **Table 4 –** Effect of male density, sib frequency (and their interaction) and the number of male
767 rejections on mating latency in *V. canescens*. We analysed the deviance table of the mixed-effect Cox
768 model with the female family as a random effect.

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	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

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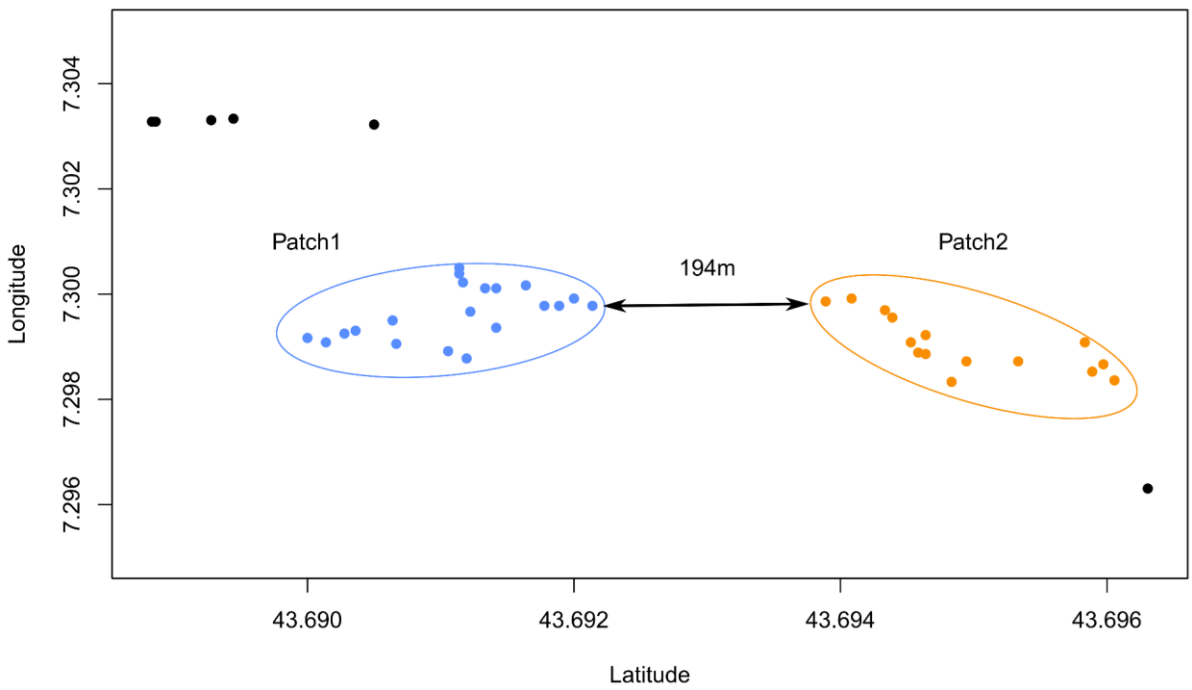

Figure 1 – Location of carob trees from the Nice field site where *V. canescens* were captured during the summer 2014. Each dot represents a tree. Axes are GPS coordinates in decimal degrees. Blue dots represent trees of "Patch 1" and orange dots represent trees of "Patch 2" (see also Table 2). Six trees (black dots) were not included because they did not fit our definition of patches.

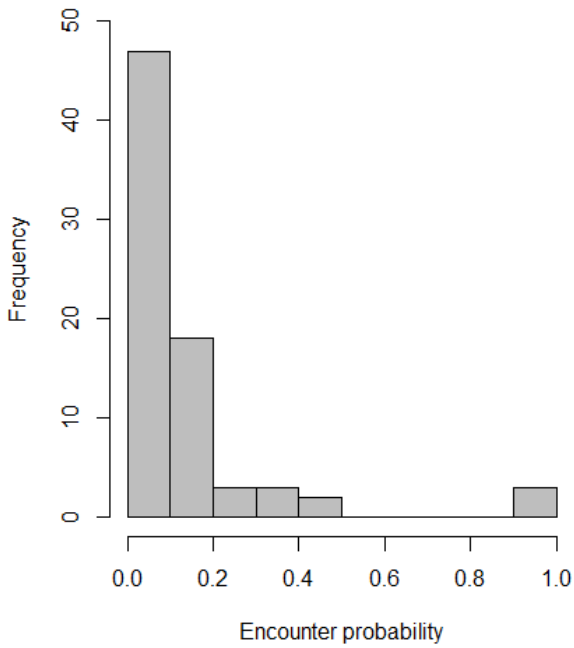

Figure 2 – Distribution of the probability that a female *V. canescens* encountered a genetically related male during the day of capture in the Nice population. For each female caught, we computed its relatedness with all males captured the same day. We then counted the number of relationships with $r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

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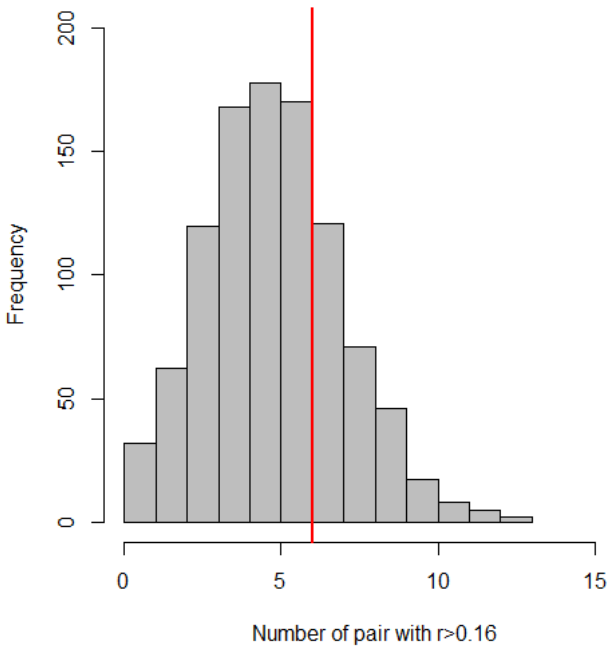

Figure 3 – Theoretical distribution of the number of crosses between genetically related *V. canescens* male and female under the assumption of random mating. The theoretical distribution was built using random sampling of a mate for each of the 51 females from the Nice population, estimating *r* for 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 $r > 0.16$.