Sexual isolation with and without ecological isolation between sympatric isopods *Jaera albifrons* and *J. praehirsuta*

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Abstract

Sexual isolation is often coupled with other barriers, and sexual isolation mechanisms can be intrinsically tied to ecological conditions or natural selection against heterospecific gene flow. The independence and relative strength of sexual isolation are thus difficult to assess. Here we take advantage of a pair of isopod species (*Jaera albifrons* and *J. praehirsuta*) that show sexual isolation and coexist in populations where they share the same habitat or not (i.e. without or with ecological isolation). Using no-choice trials and a free-choice experimental population, we estimated the strength of sexual isolation between *J. albifrons* and *J. praehirsuta* individuals originating from these different ecological contexts. We found that sexual isolation is strong in presence and absence of ecological isolation, but that it is asymmetric and fails to prevent gene flow entirely. Post-zygotic barriers were low, and there was no sexual isolation within *J. praehirsuta* across habitats. The *J. albifrons* / *J. praehirsuta* species pair thus provides an example where the role of sexual isolation as a barrier to gene flow i) does not depend upon current ecological isolation, ii) seems to have evolved independently of local ecological conditions and hybridization costs, but iii) is unable to complete speciation entirely on its own.

Key words

Speciation, asymmetric behavioural isolation, post-zygotic isolation, sexual selection, marine isopods

Introduction

Sexual isolation can evolve from sexual selection (reviewed in Coyne and Orr 2004; Ritchie 2007) but there is discussion about whether this process may ever happen independently of ecological context and natural selection (Safran et al. 2013; Servedio and Boughman 2017). This question was born not only from the fact that sexual isolation acts generally in concert with other isolating barriers but also from the realization that sexual selection and sexual isolation mechanisms are often intrinsically tied to ecological conditions (discussed in Andersson 1994; Ritchie 2007; Safran et al. 2013; Boughman and Svanback 2017; Servedio and Boughman 2017).

For one thing, species thought to have differentiated via sexual selection often coincidentally show some level of ecological isolation or other barriers. In these cases one immediate issue is to determine the relative role of sexual isolation and if it was decisive in initiating speciation. This issue centers on the initial role and present-day substantiveness of sexual isolation when it is associated with one or more other barriers between species.

Another, more intricate issue arises from the interaction that natural selection or ecological context can have on the evolution of sexual selection and sexual isolation (e.g. Ritchie 2007; Safran et al. 2013; Boughman and Svanback 2017; Servedio and Boughman 2017). It is currently unclear whether sexual selection may ever unfold into sexual isolation independently of natural selection and ecological conditions, and, pushing this line of reasoning further, one may even ask if sexual isolation can evolve independently of other barriers or if it is necessarily coupled with other barrier effects, in particular some form of ecological isolation. In a recent synthesis article, Butlin and Smadja (2018) proposed a conceptual framework to discuss how individual isolating barriers may become coupled, thereby leading to stronger reproductive isolation. This framework (outlined in Fig. 1 in Butlin and Smadja 2018) can be used to discuss the interactions between sexual isolation and natural selection or ecological context that have been the focus of much recent attention (Ritchie 2007; Safran et al. 2013; Boughman and Svanback 2017; Servedio and Boughman 2017).

First, such interactions happen when natural selection affects traits that also have a direct bearing on sexual isolation (e.g. wing color patterns affecting mimicry and mate choice in *Heliconius butterflies*, Jiggins et al. 2001). This situation is an example of a trait that contributes to two barrier effects (ecological isolation and sexual isolation), leading automatically to the coupling of these effects ("by-product coupling" sensus Butlin and Smadja 2018). Second, a similar mechanism takes place when the transmission of sexual signals involved in sexual selection and sexual isolation is directly affected by abiotic ecological conditions. In such cases the system of transmission of sexual signals may adapt to diverging environments and concurrently drive sexual isolation as a by-product (sensory-drive process, e.g. speciation in Gasterosteus sticklebacks and Pundamilia cichlids driven by adaptation of female perceptual sensitivity to ambient light combined with sexual selection on male colour, Boughman 2001; Seehausen et al. 2008). More generally, the evolution of sexual traits and preferences may be influenced by ecological conditions beyond the transmission of sexual signals (context-dependent sexual selection, see e.g. Servedio and Boughman 2017). In these cases, ecological isolation and sexual isolation evolve simultaneously, and their coupling is a by-product of this simultaneous evolution (Butlin and Smadja 2018). Finally, enhanced sexual isolation may result from natural selection when heterospecific mating incur fitness costs (e.g. local divergence in male plumage and female choice in response to selection against hybridization in *Ficedula* flycatchers, Saetre et al. 1997). This is a case of "adaptive coupling" where sexual isolation further evolved in response to another barrier (in the flycatcher example, a post-zygotic barrier).

In summary, even in cases where sexual selection effectively contributed to speciation, it may have involved tangled interactions with natural selection (reviewed in Safran et al. 2013; Servedio and Boughman 2017), and these interactions may be directly responsible for the coupling of sexual isolation with one or more other barrier effects. An objective of empirical research is thus to ask how decisive and independent is sexual isolation regarding other barriers, and to what extent did it evolve under the influence of natural selection or ecological context.

These processes seem inextricably linked, but here we take advantage of a situation where two closely related sympatric species that show strong sexual isolation generally occupy distinct habitats but can also be found in places where they coexist in the same habitat. With this situation, we cannot determine if sexual isolation initiated speciation, but we can ask if sexual isolation stands when ecological isolation doesn't, and use between-species and between-habitats experimental crosses to further ask if sexual isolation somehow evolved as a by-product of- or in response to natural selection.

We focus on crustacean isopods *Jaera albifrons* and *J. praehirsuta*, two species that belong to the *Jaera albifrons* complex. This complex is composed by five species of small (2-5 mm) marine isopods that live on the shores of the temperate and cold waters on both sides of the North-Atlantic Ocean (Bocquet 1953; Solignac 1978). All five species are phenotypically indistinguishable except for male secondary sexual traits that are used for tactile courtship (Bocquet 1953; Solignac 1981). The males of each species have specific sets of setae and spines located at different places on their peraeopods (Fig. 1) and they use these features to brush a particular region of the back of females in order to get them to engage in sexual intercourse. For instance, *J. albifrons* males have protruding lobes topped with a compact set of setae forming a brush on one segment of their sixth and seventh pairs of peraeopods. By contrast, *J. praehirsuta* males instead have more scattered setae positioned on peraeopod pairs one to four, and one or two spines on peraeopods six and seven. The three other species similarly show variations in length, shape, and position of setae and spines on male peraeopods but they all share the same basic courtship behaviour, with males mounting females in a head-to-tail position and working their brushes. Females accept or reject a male based on this tactile stimulus, and behavioural isolation is thought to ensure a nearly complete arrest of heterospecific gene flow in nature (Solignac 1978).

Sexual isolation is thus currently a very important barrier between these species, and it could have been instrumental in initiating speciation (Solignac 1981). However, the potential interactions of natural selection discussed above apply to the *J. albifrons* / *J. praehirsuta* case. First, these two

species are generally found on distinct habitats. While the two species occupy the same narrow belt of intertidal habitats along the American and European shores of the North-Atlantic Ocean and are thus found in sympatry over most of their distribution, *J. albifrons* is primarily found under pebbles and stones while *J. praehirsuta* is primarily found on intertidal brown algae (Bocquet 1953; Naylor and Haahtela 1966; Naylor and Haahtela 1967). These habitats are often in immediate proximity, and these preferences are not strict (Solignac 1981; Ribardière et al. 2017), but they still mean that ecological isolation is strong and thus its current relative contribution to total reproductive isolation must be overwhelming. Second, a post-zygotic barrier (reduction in hybrid viability and fertility after the first generation of hybridization) has been reported in the *Jaera albifrons* complex (Solignac 1976, 1981). Although this barrier has not been looked at specifically in the *J. albifrons* / *J. praehirsuta* pair, the reported genetic incompatibilities are thought to involve chromosomal differences (Solignac 1981) and such differences exist in our two focal species (Staiger and Bocquet 1956; Lécher and Prunus 1971; Ribardière 2017). Post-zygotic isolation could thus be another barrier to consider alongside ecological and sexual isolation. Third, rather than being instrumental in initiating speciation, premating isolation could have played a secondary role, evolving in response to natural selection due to hybridization costs (reinforcement of a pre-mating barrier in response to post-zygotic barrier effects, that is, adaptive coupling sensus Butlin and Smadja 2018). And finally, adaptation to life under stones or on algae could have had indirect consequences on mate choice mechanisms, somehow selecting for divergent, locally adapted, trait-preference regimes (sensory-drive process, that is, a form of by-product coupling sensus Butlin and Smadja 2018).

The *J. albifrons* / *J. praehirsuta* pair gives us an opportunity to dissect these potential interactions because the two species that usually use distinct habitats were reported to coexist in an exceptional population where they share the same habitat (under stones, that is, the primary *J. albifrons* habitat, Solignac 1969a, b). This coexistence of the two species in a unique habitat was recently found to have persisted for decades and to be more widespread than previously thought as it extends to several other sites at least in the French region Normandy and in the United Kingdom

(Ribardière et al. 2017; see also Mifsud 2011). Hybridization happens in these populations (Solignac 1969b) and results in various levels of introgression (Ribardière 2017; Ribardière et al. 2017) but most males bear sexual traits that are clearly identified as belonging to one or the other species and intermediate phenotypes are scarce.

With this system our main objective was to test whether sexual isolation stands in absence of ecological isolation (as one can hypothesize from the persistence of bimodal sexual phenotypes). If sexual isolation completely breaks down when the two species share the same habitat then it means that ecological isolation plays a major role in keeping species reproductively isolated and sexual isolation is not likely to have been as important in speciation (at least not without being coupled with ecological isolation).

Furthermore, even if sexual isolation is strong in absence of ecological isolation, it could still have had only a secondary role in speciation if it evolved or was reinforced in response to hybridization costs. Our secondary objective was thus to test whether sexual isolation is stronger in absence than in presence of ecological isolation (which would be compatible with adaptive coupling by reinforcement) and whether there are fitness costs to hybridization that could have given cause for reinforcement.

Finally, our third objective was to determine if habitats somehow interfered on the evolution of sexual isolation through a sensory drive process or other context-dependent mechanism (by-product coupling between sexual and ecological isolation). Under this hypothesis, individuals from the same species but occupying different habitats should show some level of sexual isolation.

To reach these objectives we 1) quantified sexual isolation and post-zygotic isolation between *J. albifrons* and *J. praehirsuta* using experimental "no-choice" crosses featuring individuals originating from sympatric populations with and without ecological differentiation and 2) quantified sexual isolation in a "free-choice" experiment mixing individuals from populations without ecological divergence. No-choice experiments allowed us to quantify the absolute and relative strength of several reproductive barriers in controlled conditions, including sexual isolation between otherwise

naturally segregated species. The free-choice experiment allowed us to estimate sexual isolation when there is competition between individuals, an important factor that is lacking from the no-choice approach. With this, our overarching goal was to assess the role of ecological context and natural selection in closely related species where sexual selection is thought to have had a primary role in speciation.

Methods

Study system

Contrary to males, females of the five species within the *Jaera albifrons* complex are morphologically indistinguishable. They follow the same sexual cycle (total duration ca. 3 weeks) during which embryos develop in a marsupium (brood pouch) for about 12 days (Solignac 1976). Development is direct, there is no pelagic larval stage, and offspring measure ca. 0.5 mm when they are released from the marsupium. Individuals become sexually mature and can be sexed within 4 to 5 weeks (e.g. Solignac 1979).

As mentioned in the introduction, three reproductive barriers have been identified between species of the *Jaera albifrons* group (reviewed in Solignac 1978, 1981): ecological isolation (due to habitat isolation; there is no temporal isolation), behavioural isolation (mainly driven by female mate choice based on a tactile courtship stimulus involving male secondary sexual traits), and post-zygotic hybrid sterility or inviability (essentially in F2 hybrids and backcross offspring). There is no mechanical isolation and no postmating copulatory behavioural isolation (Bocquet 1953 p. 297; Jones and Fordy 1971; Veuille 1978). Importantly, all analyses of inter-specific crosses have shown that females either rejected hetero-specific males or produced a normal number of offspring. That is, females that produced no offspring did not mate (e.g. Solignac 1978 p. 49), and females mated by a heterospecific male did not show any reduction in fecundity (e.g. Solignac 1978 pp. 80-82). One exception was observed with some crosses between males *J. albifrons* and females *J. ischiosetosa* stemming from particular populations where a deficit of female offspring was observed (Solignac

1978). The complete absence of offspring produced is thus a good indicator for sexual isolation (and most probably the behavioural component of sexual isolation, although gametic isolation has yet to be investigated in this group, see discussion).

Our study is based on the analysis of the reproductive output of virgin males and females in controlled breeding experiments (the experimental workflow is outlined in supplementary information figure S1). To obtain such individuals we first sampled (unidentified) females in natural populations with or without ecological isolation (Ribardière et al. 2017). As described in supplementary information, these populations were located either in the French region Brittany (strong ecological isolation) or Normandy (no ecological isolation). Then we individually reared in the lab the offspring produced by these females (which were fertilized by unknown males in nature prior to sampling) until they could be sexed and males could be identified. Females were sorted as *J. albifrons* or *J. praehirsuta* according to the sexual traits born by their brothers. At this stage we thus had a series of *J. albifrons* and *J. praehirsuta* virgin adults originating either from Brittany or Normandy, which we could then use in controlled experiments. Rearing conditions are described in supplementary information.

No-choice crosses

General principle and experimental set-up

In order to quantify sexual and post-zygotic isolation in presence vs. absence of ecological isolation, we ran a series of controlled crosses where one male and one female were paired and their reproductive output monitored. These crosses featured intra-specific and inter-specific crosses using either a pair of individuals from Brittany (ecological isolation) or a pair from Normandy (no ecological isolation).

In addition, we ran a series of intra-specific and inter-specific crosses pairing individuals from opposite regions (and thus opposite habitats in the case of *J. praehirsuta*, which rests on algae in Brittany vs. under stones in Normandy) in order to test for sensory-drive or other context-dependent evolution of sexual isolation. These inter-region crosses also allowed us to double-check that *J.*

albifrons (and *J. praehirsuta*) from across our two separate regions truly are the same biological species, an implicit condition of this study and most previous investigations with this pair of species (Solignac 1969b; Ribardière et al. 2017).

A total of 160 crosses were run overall (Table 1). Experimental conditions are detailed in supplementary information. For each cross we recorded i) if it successfully produced offspring, ii) how long it took for the first offspring to appear, iii) how many offspring were contained in each brood produced, and iv) offspring survival at 35 days. All data were analysed in R v.3.3.3 (R Core Team 2017) using package lme4 (Bates et al. 2015).

Estimating components of reproductive isolation

Reproductive isolation was estimated following Sobel and Chen (2014) using estimators that vary between -1 (complete disassortative mating, probability of heterospecific gene flow=1) to 1 (complete reproductive isolation, probability of heterospecific gene flow=0). To meet our goal of comparing reproductive isolation components in presence vs. absence of ecological isolation, all the computations described below were performed independently using crosses featuring individuals "from Brittany" vs. "from Normandy" (i.e. using intra-region crosses only).

First, because *J. albifrons* and *J. praehirsuta* in Brittany do not have strictly non-overlapping habitats, we used survey data from Ribardière et al. (2017) to quantify ecological isolation as

$$RI_{ecological} = 1 - (S/(S + U)) \quad (1)$$

where *U* was the proportion of individuals found on the primary habitat of their species and *S* was the proportion of individuals found on the alternative habitat (i.e. *S* is the probability that an individual is in a place where it will meet the other species, that is, "shared"). This equation gives an estimate of the reduction in heterospecific gene flow that would happen if individuals would mate randomly within each habitat (i.e. ecological isolation only).

Second, we estimated three components of sexual and post-zygotic isolation (listed in Table 2) from our experimental crosses. The strength of each reproductive isolation barrier *i* was estimated as

$$RI_i = 1 - 2\times(H_i/(H_i + C_i)) \quad (2)$$

where H_i and C_i refer to variables calculated for heterospecific and conspecific pairs (Sobel and Chen 2014). For sexual isolation (RI_1), *H* and *C* were the proportions of inter-specific and intra-specific crosses that successfully produced offspring. For components of post-zygotic isolation, *H* and *C* referred to brood size (number of offspring) or survival (proportion of offspring surviving at day 35) observed from intra-specific crosses and inter-specific crosses (see column "parameter" in Table 2). Each of these components of reproductive isolation was thus estimated independently, giving the strength RI_i that each barrier would have if it were acting alone.

Total reproductive isolation was estimated using the product of H_i and C_i across all components of isolation (noting that we used multiplicative components of fitness: probability of encounter, probability that a cross is successful, brood size, and probability of offspring survival). Heterospecific encounters can only happen in a "shared" ecological context, so H_i values were defined only under the condition that heterospecific individuals meet in nature (and this happens with probability *S*). Conspecific encounters happen in any context (shared or not), but C_i could theoretically take different values (i.e. conditionnal on *S* and *U*, Sobel and Chen 2014). Here because we used no-choice trials, we took C_i values to be equal in any ecological context (e.g. we considered the probability that a conspecific cross was successful to be independent on whether such an encounter would happen in a shared or an unshared context in nature). Total reproductive isolation was therefore calculated as:

$$RI_{tot} = 1 - 2\frac{S\times\prod_i H_i}{S\times\prod_i H_i + \prod_i C_i} \quad (3)$$

Finally, we estimated the absolute contribution of each individual barrier by subtracting the effect of previously acting barriers as (Sobel and Chen 2014):

$$AC_i = RI_{[1,i]} - RI_{[1,i-1]} \quad (4)$$

With this definition, individual contributions AC_i can be seen as additive components of RI_{tot}.

Free-choice experiment

General principle and experimental set-up

255 The general principle of the free-choice experiment (Fig. 2) was to estimate sexual isolation based on the reproductive output of males and females *J. albifrons* and *J. praehirsuta* freely interacting in an experimental population (and thus experiencing intra-sex competition and easier male avoidance by females, unlike in no-choice experiments). This experiment focused only on the situation where the two species occupy the same habitat in the wild and thus interact all the time.
260 Hence we used virgin adult individuals obtained from the no-choice crosses described above (region Normandy only), so that we could mix *J. albifrons*, J*. praehirsuta*, and F1 hybrids all obtained in the same controlled conditions and all originating from a region without ecological isolation (Fig. S1).

We chose 15 females of each species, 17 males of each species, and 17 males produced by inter-specific crosses. These numbers were constrained by several parameters, including the fact that
265 we avoided mixing related males and females (i.e. two males could be brothers, but we did not pick males and females from within the same family). A mixture of 30 virgin females and 51 virgin males of controlled origin therefore composed our experimental population (Fig. 2). We will refer to these individuals as the "parents" (or parental generation), themselves produced in the lab from known mothers and fathers that we will call "grandparents" when needed.

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Here we outline the experimental set-up, which is presented into more detail in supplementary information. All adults were put together in a small aquarium for 12 days. This is the minimum time required for a female to produce offspring if such a female would have been fertilized early in the experiment (Solignac 1976). All surviving females (n=27 females) were then removed
275 from the aquarium and placed individually in 6-well plates that were then checked for offspring 2 to 3 times per week for 66 days.

Offspring were observed between 3 to 66 days after females were isolated (only one female alive after 66 days had not produced offspring, see results). During that time all the offspring

produced were isolated and reared individually for at least 57 days (see conditions in supplementary information). They were then sexed, photographed, and fixed in ethanol. In addition, the secondary sexual traits of all male offspring (Fig. 1) were examined under a microscope. All potential parents were also fixed in ethanol (after examination of the sexual phenotype of males).

Female identification and parentage assignment

We used genetic parentage analyses to achieve two goals. First, while the females that entered the experimental population were identified as *J. albifrons* or *J. praehirsuta* based upon the type of controlled cross from which they originated, they could no longer be identified once they were mixed up altogether and later isolated one by one until they produced offspring (because the females of each species are nearly morphologically identical). Hence we used DNA data from the individuals that produced these females (the "grandparents" used in controlled crosses previously performed in the lab) in order to re-identify each female through genetic parentage assignment. Female identification was aided by photographs taken before and after the experiment (see supplementary information). Second, we used parentage analyses to identify the fathers that produced each offspring (the mothers were known since females were kept individually until they produced offspring, Fig. 2).

To achieve these goals, all offspring, potential parents, and grandparents were genotyped at 13 microsatellite loci (Ja02, Ja13, Ja21, Ja22, Ja23, Ja30, Ja35, Ja39, Ja41, Ja64, Ja71, Ja78, and Ja94, Ribardière et al. 2015). DNA extraction, PCR amplification, and allele scoring were performed following Ribardière et al. (2015). Multilocus genotypes were then used for parentage assignment in the software Colony v2.0.6.1 (Jones and Wang 2010) as detailed in the supplementary information.

Sexual isolation

We aimed to compare sexual isolation in these free-choice settings with that measured in no-choice crosses. Hence in a first step we used the same theoretical framework (Sobel and Chen 2014)

305 to estimate sexual isolation RI_1 following equation (2) with H_1 and C_1 defined as the proportions of inter-specific and intra-specific crosses that successfully produced offspring. These proportions were calculated as the number of successful pairs divided by the number of potential pairs that could possibly have formed. Note that because we initially introduced the same number of males of each species in the experiment, using proportions (as above) or absolute numbers (as in Sobel and Chen

310 2014) of successful pairs would lead to the same result for RI_1. It turned out that sexual isolation was very strong: only one interspecific pair and two pairs involving F1 hybrid males produced offspring, while all other successful pairs were conspecific (see results). Hence downstream barriers involving brood size and survival were not quantified because they would be based on too few samples.

In a second step, sexual isolation was estimated using a framework described by Rolan-Alvarez

315 and Caballero (2000) that applies to multiple-choice experiments. While this estimation procedure gives a less direct estimate of heterospecific gene flow reduction than Sobel and Chen's method and cannot be applied to our no-choice experiments, it has several interesting properties. In particular, it takes into account inequalities in mating frequencies rather than assuming that the two species have the same propensity to mate, and it can detect asymmetry in sexual isolation.

320 To estimate sexual isolation we counted the number of male/female pairs of each type (e.g. *J. albifrons*/*J. albifrons, J. albifrons*/*J. praehirsuta*, etc.) that successfully reproduced. Pair sexual isolation (*PSI*) was estimated for every pair type following Rolan-Alvarez and Caballero (2000). This method gives a conservative view of sexual isolation as it is defined for each pair type as the number of observed pairs divided by the number of expected pairs given the actual mating success observed

325 We then followed these authors' recommendation to estimate I_{PSI}, a modified joint isolation index (Merrell 1950) based on *PSI* statistics and that varies from 0 (no isolation) to 1 (complete isolation). Details of the computation are described in Rolan-Alvarez and Caballero (2000) and Perez-Figueroa et al. (2005). Values of *PSI*, I_{PSI}, and their statistical significance were computed using JMating v1.0.8 (Carvajal-Rodriguez and Rolan-Alvarez 2006).

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Mating success, reproductive success, and male phenotypes

Mating success (number of mates of each adult male and female in the experimental population) and reproductive success (total number of offspring parented by each adult) were estimated directly from the results of the parentage analyses. That is, these analyses are based on the identity of offspring that survived up to adulthood (57-70 days). Since females were reared individually until they produced offspring, their fecundity was also estimated as the number of offspring that they released (including offspring that later died during the experiment and could not be assigned a father).

Male phenotypes were summarized using principal component analyses (PCA) based upon the following 13 phenotypic variables: carpal lobe on peraeopods 6 and 7 (P6 and P7) coded as 0 (absent), 0.5 (small), or 1 (large), number of spines on P6 and P7, total number of straight or curved setae on the carpus of P6 and P7, number of distal setae on P6 and P7, and total number of curved setae on peraeopods P1 to P5 (i.e. adding setae counted on each segment). The PCAs were run in R v.3.3.3 (R Core Team 2017) using package ade4 v.1.7.6 (Chessel et al. 2004). This approach produces linear combinations of traits that are more efficient for investigating sexual isolation than multiple trait variables separately (Hohenlohe and Arnold 2010). Finally, we used coordinates on the first PCA axis to test whether the probability that a female of one or the other species will mate with a male matched the distribution of male sexual traits of the corresponding species (building upon Arnold et al. 1996).

Results

No-choice experiment

Raw data

Crosses were informative only if the female survived for long enough to have a chance to produce offspring. To avoid false negatives, we removed from the analyses all crosses (*n*=18) for

which the female survived for less than 24 days after being placed with a male (this threshold was chosen because there was only one successful cross with a female that survived for a shorter time).

Reproductive output from experimental crosses

360 Out of the remaining 142 crosses, 77 successfully produced offspring. As shown in Figure 3, intraspecific crosses were more successful (63 out of 78) than interspecific crosses (14 out of 64). This difference is significant whether one is looking at all data or focusing on data from the region with ecological isolation (Brittany), without ecological isolation (Normandy), or inter-region crosses (Fisher's exact tests, all p-values < 0.025). Interspecific crosses were generally not very successful, but 365 two tendencies can be noted: they seemed slightly more successful in Normandy than Brittany (Fig. 3), although this was not significant (p=0.51), and they were more successful when a pair's female was *J. praehirsuta* rather than *J. albifrons* (Fig. 4, p=0.03).

Focusing on inter-region crosses, we found the success of intra-specific crosses to be high (28 out of 35 crosses produced offspring) and similar to that of intra-specific crosses within each region 370 (Fig. 3, Fisher's exact test p=0.33). Moreover, this high success was equal for *J. albifrons* pairs and *J. praehirsuta* pairs (respectively 15 out of 19 and 13 out of 16 crosses produced offspring, p=1). That is, there was no difference in success when a male and a female came from the same *vs.* different regions, and this was true for each of the two species.

Not only were intraspecific crosses more successful, but they also produced offspring more 375 rapidly (40.33 days on average, Fig. 5) than interspecific crosses (62.1 days, generalized linear model GLM with quasi-Poisson family p < 0.001). The time needed to produce offspring was also much more variable among interspecific crosses (Fig. 5). These patterns were consistent across treatments (within each regions and inter-regions, data not shown). There were too few interspecific crosses with *J. albifrons* females to test for a difference in the time to offspring production between 380 reciprocal interspecific crosses.

Successful broods contained from 1 to 33 offspring (8.7 ind. on average, Fig. 6). Brood size did not differ significantly (GLM quasi-Poisson family, p=0.22) between intraspecific (average 8.3 ind.) and interspecific crosses (10.5 ind.).

On average 80.4 % of the offspring were still alive at day 35 (Fig. 7). There was no difference in survival between intraspecific (80.2%) and interspecific crosses (81.2%, GLM quasi-binomial family p=0.85).

Brood size did not differ between intraspecific and interspecific crosses, and abortion of an entire brood of embryos was never observed (oocytes and developing embryos are visible in the marsupium of females). These results confirmed that females in presence of a heterospecific male either produced no offspring because they did not mate, or produced a normal number of offspring. The success of crosses (presence vs absence of juveniles produced) is thus a good indicator of sexual isolation.

Reproductive isolation estimates

All estimates of reproductive isolation are presented in Table 2. Ribardière et al. (2017) reported for region Brittany 141 *J. albifrons* under pebbles *vs.* 2 on algae, and 3 *J. praehirsuta* under pebbles *vs.* 77 on algae. Ecological isolation can thus be estimated to 98%. Because this reproductive barrier is the first to occur in real world conditions, all other barriers will have very little effect in natural populations in this region. This reflects in the low values for absolute contributions (AC_i column in Table 2). No-choice experiments revealed however that sexual isolation was strong ($RI_{1,Brittany}$= 0.73, that is, it would reduce heterospecific gene flow by about 70% in situations were individuals of the two species would meet, as in the case for instance when bold *J. albifrons* individuals venture on algae, or *J. praehirsuta* on pebbles). Post-zygotic components of isolation were low.

By contrast, there was no ecological isolation in mixed *J. albifrons* / *J. praehirsuta* populations from Normandy (Ribardière et al. 2017) and there we find sexual isolation ($RI_{1,Normandy}$= 0.37) to be

half of that found in Brittany. Other components of reproductive isolation were negligible (Table 2). In this region sexual isolation is the first barrier to occur, hence it effectively leads to a strong reduction in heterospecific gene flow ($AC_{1,Normandy} = RI_{1,Normandy} = 0.37$), by contrast with the situation described above for Brittany where sexual isolation was twice as strong ($RI_{1,Brittany}$= 0.73) but had very little effect in nature ($AC_{1,Britanny}$ = 0.015, Table 2).

Free-choice experimental population

Raw data

Out of 30 potential parent females, 7 died during the experiment (no offspring produced), 22 produced offspring (14 *J. albifrons* and 8 *J. praehirsuta*), and the last one (*J. praehirsuta*) was watched for 66 days (once pulled out from the experimental population) but did not produce any offspring.

Out of 51 males, 1 male was found dead one day before females were removed from the experimental population, and 2 were never found (and thus could have died early in the experiment without being detected). But 2 out of these 3 males actually did sire offspring. So it leaves a maximum of 1 male out of 51 for which we don't know if it died too early to have a chance to court females. We considered that this uncertainty was negligible (that is, all further computations and interpretations are based as if all males had equal chances to reproduce).

More importantly, the parentage analyses revealed two errors in the experimental setup. First, a set of four brothers that were thought to originate from an interspecific controlled cross ("grandparents": female *J. praehirsuta* / male *J. albifrons*) in fact did not originate from this cross. Before being taken out and used in the controlled cross, their mother had mated with one of her brothers that managed to crawl between wells in the 6-well plate where this family was raised (i.e. just before step 2 in Fig. S1). Fortunately, these four males (inbred *J. praehirsuta* instead of "hybrids") did not sire any offspring in the experimental population and they were simply removed from all downstream analyses. The second error is similar: the very first female that produced

offspring after being taken out of the experimental population in fact had been mated by one of her brothers before the experiment (also because that male got out of its well and went into that of her sister, i.e. just before step 3 in Fig. S1). Hence this female and her brood were also taken out from all downstream analyses. No other issues were detected: parentage analyses showed that all other parents corresponded to our controlled crosses and all other offspring were conceived in the experimental population.

In summary, all downstream analyses were based on reproduction patterns for 47 males and 22 females. These individuals produced 359 offspring, out of which 239 could be genotyped (that is, offspring that survived for 57-70 days until they were fixed in alcohol or were found dead in the course of the experiment but could be correctly genotyped). Overall, 5 females were kept alive until they produced a second brood (3 *J. praehirsuta* and 2 *J. albifrons* females, 1 to 17 offspring per brood). Each of these second broods were parented by the same single male as the first broods, so that these second broods did not affect our estimates of mating success. As mentioned in Methods, these second broods were removed from all downstream analyses, and reproductive success measures were finally based on 218 offspring stemming from the first brood of each female parent.

Sexual isolation

A total of 26 male/female pairs produced offspring. As it turned out (Fig. 8), *J. albifrons* females reproduced only with *J. albifrons* males (*n*=18 pairs), while *J. praehirsuta* females reproduced with the three types of males: *J. praehirsuta* males (*n*=5), "hybrid" males, (*n*=2), and *J. albifrons* males (*n*=1). This distribution is not random (Fisher's exact test for independence between male and female species composing these pairs p<0.001) and leads to an estimate for sexual isolation between *J. albifrons* and *J. praehirsuta* equal to $RI_{1,exp.pop.}$=0.92 (CI_{95} [0.75;1]), a number that can be directly compared with $RI_{1,Normandy}$=0.37 obtained from no-choice crosses.

These results assume that all individuals had the same probability to mate. Rolan-Alvarez and Caballero's method based on the calculation of pair sexual isolation (*PSI*) is much more conservative,

since it takes into account the effect of differences in mating success between individuals from each species (e.g. here *J. albifrons* individuals mated more frequently than others). Figure 8 shows the number of mating pairs of each possible type expected from random mating in absence of sexual isolation but taking into account the variation in mating frequencies. Estimates of *PSI* are given in Table S1. As explained by Rolan-Alvarez and Caballero (2000), values of *PSI* above/below one indicate excess/deficit of observed pairs relative to expected pairs under random mating using marginal frequencies. The global modified joint isolation index I_{PSI} (Rolan-Alvarez and Caballero 2000) was equal to 0.46, while pair-specific values were $I_{PSI}^{albi/prae}$=0.92, $I_{PSI}^{albi/hybrid}$=1, and $I_{PSI}^{prae/hybrid}$=0.38, indicating strong isolation between *J. albifrons* and *J. praehirsuta*, and lower isolation between *J. praehirsuta* and hybrids. Bootstrap-based tests performed in JMating for all *PSI* and I_{PSI} estimates showed that only $I_{PSI}^{albi/prae}$ was statistically significant (*p*=0.004).

Male and female reproductive success

Out of 22 females, most (16) mated with a single male, while 5 had 2 mates and 1 had none (Fig. S3A). By contrast, the majority of males had no success (31 out of 47), while other males mated with 1 to 4 female partners. There were no obvious differences in reproductive success distribution between species (Figs. S4 and S5).

J. albifrons females produced from 3 to 31 offspring per brood (*n*=14, mean=16.64), while the fecundity of *J. praehirsuta* females seemed slightly lower (5 to 19 offspring per brood, *n*= 7, mean=11.71, excluding the one female that produced no offspring at all), although this difference was not significant (*n*=21, generalized linear model *p*=0.146). Female fecundity was significantly related with body size (*n*=18 females for which we had body size at the time when the first brood was released, generalized linear model *p*=0.022). There is little power to test this, but the three *J. praehirsuta* females that mated with heterospecific males did not show any sign of reduced fecundity (brood size 7, 15, and 19 offspring, and the female that released only 7 offspring in her first brood produced a second brood of 17 with the same heterospecific father).

485

Polyandry and multi-paternity

As mentioned above, 5 females had 2 male partners (4 out of 14 reproductive *J. albifrons*, and 1 out of 7 reproductive *J. praehirsuta*). The four *J. albifrons* females mated with two distinct *J. albifrons* males, while the *J. praehirsuta* female mated with one *J. praehirsuta* and one *J. albifrons* male. All 5 cases corresponded to multipaternity within the first brood (and not to two different fathers in two different broods). These broods contained 15 to 23 offspring (7 to 10 genotyped offspring), and the minimum contribution of a given father was 0.14.

Male secondary sexual traits

Regarding the parental generation, we described the phenotype of 15 *J. albifrons*, 15 *J. praehirsuta*, and 13 hybrid males, that is, 43 of the 47 males that entered the experimental population (excluding the four males that were discarded from further analyses). We also described the phenotype of 137 males from the offspring generation. Phenotypic variability was large both in parents and offspring, and it included phenotypes that are typical of *J. albifrons*, *J. praehirsuta*, and intermediate phenotypes (Table S2). This variation can be visualized from principal component analyses (Figs. 9 and S6). The first two PCA axes explained 61.8% and 18.3% of the variation in parent phenotypes, and 59.6% and 14.9% of the variation in offspring phenotypes (male secondary sexual traits). Using PCA axis 1 to summarize male sexual traits, we see in Figure 9 that the preferred mates of *J. albifrons* females coincide with the distribution of *J. albifrons* male sexual traits, while *J. praehirsuta* females mated with males showing a wider range of trait values.

Discussion

One of the questions that hang over the speciation-by-sexual-selection process is to what extent sexual selection and its consequences on sexual isolation depend upon ecological context and natural selection (discussed in Andersson 1994; Ritchie 2007; Safran et al. 2013; Boughman and

Svanback 2017; Servedio and Boughman 2017). Even in cases where sexual selection effectively contributed to speciation, it may have involved tangled interactions with natural selection, and these interactions may be directly responsible for the coupling of sexual isolation with one or more other barrier effects. Here, we dissected these potential interactions in a pair of species for which sexual isolation is thought to have been a key component of speciation but that also differ in habitats. We found that sexual isolation is strong both in presence and absence of ecological isolation, but that in both cases it is asymmetric and fails to prevent gene flow *entirely* on its own. We also found that sexual isolation mechanisms do not seem to be intrinsically tied to local contexts. We discuss below how these findings make one step towards understanding the origin of sexual isolation in this system, which we think has evolved independently of ecological conditions but did not suffice to fully complete speciation.

Sexual isolation is strong but not sufficient to prevent heterospecific gene flow in absence of ecological isolation

Reproductive isolation estimates from no-choice trials (Table 2) told us that sexual isolation was stronger (possibly twice stronger) in populations where the two species are ecologically separated ($RI_{1,Brittany}$= 0.73, CI_{95} [0.43-1]) compared with populations where the two species dwell in the same habitat ($RI_{1,Normandy}$= 0.37, CI_{95} [0.06-0.73]). However, in experimental conditions where males compete for access to females (at least through female mate choice, if not through direct interactions) and females can more easily escape males, we found sexual isolation in absence of ecological isolation to be as high as 0.92 (CI_{95} [0.75-1]). We conclude that sexual isolation in natural populations is most likely very strong whether or not the two species share the same habitat. This is not proof that sexual isolation was instrumental in initiating speciation, but it tells us that the role of sexual isolation as a barrier to gene flow does not depend upon ecological isolation.

535 But strong does not mean strict, and we found sexual isolation to be below 100%. Hence even if sexual isolation is strong independently of ecological isolation, it is not sufficient to prevent heterospecific gene flow entirely on its own.

The primary conclusion of this study is thus that sexual isolation plays a primary role in keeping *J. albifrons* and *J. praehirsuta* apart (it is not secondary to ecological isolation) but it cannot maintain
540 species isolation entirely without other isolating barriers.

Sexual isolation did not evolve from reinforcement in shared habitat

Sexual isolation was as strong (or even stronger according to the no-choice trials, Table 2) where species are ecologically isolated and thus have little opportunity for hybridization. This pattern
545 goes against the hypothesis that sexual isolation in *J. albifrons* and *J. praehirsuta* increased in response to selection against hybridization in places where the two species occupy the same habitat. Hence we see no sign of reinforcement, a form "adaptive coupling" where sexual isolation would have evolved in response to post-zygotic barriers.

In line with this result, we found no sign of post-zygotic isolation from our no-choice
550 experiments (AC_2 and AC_3 added to -0.04 and -0.06 in regions Brittany and Normandy, Table 2). We could not quantify post-zygotic isolation from the free-choice experimental population because only one heterospecific mating pair and two backcrosses (F1-hybrid fathers) produced offspring. Yet these broods showed no sign of reduced fitness in any way (brood size and offspring survival, data not shown). Our experiments thus did not reveal any hybridization cost that could spring reinforcement
555 in mixed populations. However, two caveats must be noted for these measurements: first, because sexual isolation was globally strong, it follows that some of the downstream barriers were estimated using small samples (e.g. only 2 interspecific broods were obtained using individuals from region Brittany, meaning that brood size for heterospecific broods was estimated for only 2 broods in that particular region). Second, and more importantly, there are many ways in which hybridization could
560 be costly (Servedio and Noor 2003) and here we estimated only a subset of these possibilities (F1

hybrid inviability and sterility), and only in lab conditions. Past research with a different pair of species (Jaera albifrons and J. ischiosetosa, which are more divergent than our focal species J. albifrons and J. praehirsuta, Ribardière 2017) has found that post-zygotic barrier effects act after the first generation (back-crosses or F2 hybrids). In a complementary study aiming at QTL detection, we ran a series of 30 backcrosses using the F1 individuals reared for the present analysis. While this was not the main focus of our work and the protocol differed slightly (two females placed with one male), we used this data to estimate three aspects of post-zygotic isolation acting after the first-generation (proportion of successful crosses, number of offspring released per brood, and survival probability at 35 days), finding no sign of post-zygotic isolation (the sum of these three potential barrier effects added to -0.06 and 0.02 for regions Brittany and Normandy, respectively, data not shown).

It is still possible that backcross offspring develop less rapidly or that F1 hybrid males experience some partial behavioural sterility (as suggested by their relatively low success in our free-choice population, where 2 out of 13 such males mated). F1 hybrid females on the other hand obviously did not suffer from such problem (RI_4 was negative in both regions, Table 2, and hybrid females produced offspring more rapidly than any other female, Fig. 5). In conclusion, while the fitness consequences of hybridization have not been fully assessed, we did not find any pattern in support for an adaptive coupling between sexual isolation and post-zygotic barriers.

Did sexual isolation evolve independently of ecological contexts?

J. praehirsuta individuals in Brittany *vs.* Normandy dwell on different habitats (pebbles vs. brown algae). But intraspecific crosses had exactly the same success (and delay to offspring production) whether or not males and females originated from the same region or different regions. And this success was the same as that of *J. albifrons* pairs (within or across regions). Hence habitat differences did not induce any sign of sexual isolation between individuals originating from algae *vs.* rock populations in species *J. praehirsuta*. Sexual isolation was unaffected by the habitat of origin and thus we can conclude that it did not evolve through sensory drive or other context-dependent

sexual selection mechanism that would have selected for divergent, locally adapted, trait-preference regimes. In other words, we found no sign of a by-product coupling between sexual isolation and ecological isolation due to context-dependent sexual selection mechanisms.

However, to suit our purpose of estimating reproductive isolation in different populations, all individuals were reared in identical, artificial, lab conditions (see supplementary information) where all five species of the *Jaera albifrons* complex had been shown to have high fitness (Bocquet 1953; Solignac 1978). This means in particular that all individuals used in cross experiments were born and raised in the exact same conditions, regardless of their population of origin. Hence we cannot exclude that contrasted substrates (algae vs. rocks) could have some immediate effect on mate choice mechanisms, for instance through phenotypic plasticity in epicuticular hydrocarbons or other contact chemical cues involved in tactile courtship (e.g. Stennett and Etges 1997). Such chemical signalling mechanisms have not yet been investigated in the *Jaera albifrons* complex.

In addition, as mentioned above we observed from no-choice trials that sexual isolation was twice as strong in populations where the two species are ecologically separated (note that all individuals were reared in the same lab conditions for these experiments as well). One plausible explanation is that mate choice is slightly relaxed in introgressed populations, but only in situations where mate options are scarce (in agreement with our finding that sexual isolation was stronger in free-choice experimental population). It is often argued that this could happen in the wild when strongly dynamic habitats (such as shores where pebbles and stones are more or less covered over by sand from one season to another) modulate the intensity of sexual isolation by limiting mate choice options (perhaps in some cases approaching no-choice conditions, if for example a female of species A finds herself isolated with males of species B only).

In conclusion, while it seems unlikely that sexual isolation has evolved in response to- or as a by-product of ecological constraints, we cannot exclude that local ecological conditions could have some subsequent effect on behavioural isolation.

Sexual isolation is asymmetric

Results from the experimental population showed sexual isolation to be strongly asymmetric (Figs. 8 and 9). In this experiment, *J. albifrons* females mated exclusively with *J. albifrons* males while *J. praehirsuta* females mated with *J. praehirsuta* (*n*=5), *J. albifrons* (*n*=1) and F1 hybrid males (*n*=2). Accordingly, pair sexual isolation indices (Table S1) for heterospecific pairs were smaller (indicating stronger isolation) when the female involved was *J. albifrons* rather than *J. praehirsuta*. Summarizing male sexual phenotypes using the first axis of a principal component analysis (Fig. 9), we found that the probability that a male mated with a *J. albifrons* female matched the density distribution of *J. albifrons* male sexual traits. By contrast, the probability that a male mated with a *J. praehirsuta* female was clearly less concordant to the density distribution of *J. praehirsuta* male sexual traits (Fig. 9B).

This finding is also confirmed by the no-choice experiments, where inter-specific crosses were systematically more successful with *J. praehirsuta* females (this is true with crosses within Brittany, Normandy, and inter-regions). Over all interspecific crosses, 4 out of 36 were successful when the female was *J. albifrons*, while 10 out of 28 were successful when the female was *J. praehirsuta* (Fisher's exact test p=0.036). Interestingly, the same result was obtained by M. Solignac with no-choice experiments using individuals from a population from France (Brittany) crossed with individuals from Germany (0% success for German *J. albifrons* females crossed with French *J. praehirsuta* males vs 22 to 46% success for French *J. praehirsuta* females crossed with German J. *albifrons* males, depending on the method of calculation, Solignac, 1978).

The asymmetry in sexual isolation is thus observed in all conditions: large-scale allopatry (between countries, Solignac 1978), smaller-scale allopatry (between regions, this study), sympatry with ecological isolation and no hybridization (Brittany, this study), and sympatry without ecological isolation and with introgressive hybridization (Normandy, this study). This is interesting because it suggests that the asymmetry has a general cause of ancestral origin and has not evolved in response to local conditions, in agreement with our previous conclusions that sexual isolation has not evolved

following sensory-drive or reinforcement processes. The many forces that can potentially cause an asymmetry in sexual isolation (discussed e.g. in Svensson et al. 2007) are tied to the evolution of sexual isolation itself, which is discussed below.

Did sexual isolation between *J. albifrons* and *J. praehirsuta* evolve from sexual selection?

We follow Coyne and Orr's definition of sexual isolation (Coyne and Orr 2004, p. 213 and Table 1.2 p. 28-29): it can include behavioural isolation and different elements of copulatory and gametic isolation. We know from previous work that behavioural isolation is the major component of sexual isolation in the *Jaera albifrons* group, and that there seems to be no copulatory (Bocquet 1953, p. 297-298) or non-competitive gametic isolation (Solignac 1978, p. 80-82). It is totally unknown, however, whether competitive gametic competition happen. We have shown in this study that females can store sperm from at least two males for several months, which may set the stage for sperm competition and/or cryptic female choice. This aspect deserves further empirical investigation, and we discuss below only the evolution of the behavioural component of sexual isolation (see Coyne and Orr 2004, p. 217 for a review of the forces that can cause the evolution of behavioral isolation).

Male sexual traits in *J. albifrons* and *J. praehirsuta* are used only for courtship behaviour and bring no other direct benefit to the bearer (e.g. advantage in local adaptation, Tinghitella et al. 2009; or in male-male competition, Sefc et al. 2015), and there are also no obvious direct benefits or costs for choosers (e.g. resource provided by mating partners, or, on the contrary, detrimental effects of mating, e.g. Svensson et al. 2007). The differences between males also involve clearly distinct, non-overlapping characteristics that are not compatible with a loss of courtship elements in one of the two species (Kaneshiro 1980; Ryan and Wagner 1987). In addition, in this study we suggested that sexual isolation did not evolve alongside particular ecological conditions (sensory-drive) or in response to hybridization costs (reinforcement). These observations together eliminate many potential evolutionary forces, leaving models of sexual selection involving indirect benefits for

665 female choice (e.g. Fisher-Lande process, or good- or compatible genes models) as the most plausible driver of sexual isolation between *J. albifrons* and *J. praehirsuta*. Under this hypothesis, sexual selection would also have driven the asymmetry in sexual isolation.

This hypothesis is based on indirect deductions and will now need empirical testing. We emphasize, however, that the patterns of reproductive success observed in this study bring some
670 early support to this proposition. Using data from our experimental population (detailed in supplementary information) we found that opportunity for sexual selection I_s (variance in relative mating success, Arnold and Wade 1984) was large for males ($I_{s,m}$=3.24, Table S3) and a male's total reproductive success (total number of offspring sired) was significantly related with their mating success (relative Bateman gradient across all male types β_{ss}=0.97, linear regression p<0.001, Table
675 S3 and Fig. S7). In absolute numbers, it means that each additional female partner allowed a male to sire 8 additional (surviving) offspring on average (Fig. S7). An upper bound for the intensity of sexual selection during a sexual selection episode can then be calculated as $s'_{max} = \beta_{ss}\sqrt{I_s} = 1.75$ (following Jones 2009). This result is consistent when one looks separately at *J. albifrons* and *J. praehirsuta* males (s'_{max}=1.19 and 1.28, respectively, Table S3), and it is largely relevant of sexual
680 selection within species (because only one heterospecific *J. albifrons* / *J. praehirsuta* mating occurred). These findings tell us that all the necessary ingredients for sexual selection are there, and that sexual selection could be quite strong (Jones' index is highly correlated with the strength of sexual selection in simulated systems, see Henshaw et al. 2016 for a range of examples).

685 Why do *J. albifrons* and *J. praehirsuta* coexist in hybridizing populations?

Individuals identified as *J. albifrons* in region Brittany (with ecological isolation) belong to the same biological species as individuals identified as *J. albifrons* in region Normandy (where there is no ecological isolation), and likewise for species *J. praehirsuta*. This was argued by taxonomists and evolutionary biologists based on the observation of secondary sexual traits (Bocquet 1953; Prunus

690 1968; Solignac 1978) and confirmed in this study using cross experiments: conspecific crosses gave the same results within and across regions (no reproductive isolation between regions).

However, we also know that reproductive isolation is not complete in populations without ecological isolation, where hybridization leads to introgression (Bocquet and Solignac 1969; Solignac 1969b, 1978; Ribardière et al. 2017). This situation raises questions about the conditions of
695 coexistence of the two species in spite of hybridization on the one hand, and ecological equivalence on the other. Ribardière et al. (2017) discussed the peculiar nature of the hybridizing populations, emphasizing that they seem to receive no influx of individuals from pure parental populations, and that no fine-scale ecological differentiation was observed (the two species are repeatedly found to be intermingled in a number of different sites). The authors suggested sexual and post-zygotic
700 isolation as two potential forces somehow acting to keep the species isolated. Here we found sexual isolation to be effectively strong in hybridizing populations, but very little support for post-zygotic mechanisms. This means that sexual processes (mate choice and/or intra-sex interactions) constitute the only force identified so far that could maintain the two species separated in spite of extensive introgression. This alone is unusual, and the fact that the two species are found to coexist in different
705 places and on the long term despite ecological equivalence poses another conundrum that deserves further investigations.

Conclusion & perspectives

In this study we looked at two closely related species that are reproductively isolated by
710 ecological and sexual barriers, asking first if sexual isolation could maintain species integrity on its own or is only acting secondarily alongside ecological isolation, and second if sexual isolation may have evolved independently of local conditions (ecological context and local natural selection for increased pre-mating barriers). We found that sexual isolation is a strong barrier that does not fall apart when ecological isolation is absent, thereby indicating that it cannot be ruled out as a primary
715 driver of reproductive isolation. And we found that sexual isolation most probably evolved

independently of local ecological conditions and local hybridization context. These results suggest that the *J. albifrons* / *J. praehirsuta* pair is an example where sexual isolation evolved and acts largely independently of other forces (at the exception, perhaps, of local ecological disturbance that could modulate mate choice options through variations in density). Yet, sexual isolation alone falls short of ensuring total reproductive isolation, and ecological isolation seems to be necessary for reproductive isolation to be complete.

This pair of species shows many indirect clues suggesting that sexual isolation evolved directly from sexual selection, but whether or not it initiated speciation remains an open question. One way forward with this question will be to assess whether replicates of mixed populations occupy a range of different habitats. If sexual isolation did drive speciation, one predicts to find the *J. albifrons* / *J. praehirsuta* pair in different habitat settings (the strongest evidence would come from populations where the two species have inversed ecological niches compared with the ones that have been described so far). Given the extended geographic distribution of these two species (American and European coasts of the temperate and cold waters of the Northern-Atlantic Ocean), this prediction is testable. Finally, the conditions of coexistence of these two species in hybridizing populations are another open question. For that, further fine-scale tests for habitat specialization (unseen so far) or interactions favouring for instance density-dependence mechanisms (pathogens?) may provide some answers.

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Author contributions

AR and TB designed the study and participated to all aspects of its realization and interpretation; EP performed most of the free-choice experiment; EP, JC, CH and SL designed and realized field sampling and species identification; EP, JC, CH, and SH designed and performed crossing experiments and phenotype descriptions. AR, EP, and CDT performed the genotyping analyses. TB wrote the article, with revisions by all other authors.

Conflict of interest disclosure

The authors of this preprint declare that they have no financial conflict of interest with the content of this article

Data accessibility

Data will be archived in Dryad digital repository upon acceptance

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Table 1 - Number of experimental "no-choice" crosses performed in order to estimate reproductive isolation between marine isopods *J. albifrons* and *J. praehirsuta* in two French regions where they live in distinct habitats (Brittany) or the same habitat (Normandy).

Cross type	Female	Male	Intra-region		Inter-region	
			Brittany	Normandy	Britt. fem.†	Norm. fem.‡
Intra-specific						
	J. albifrons	*J. albifrons*	17	6	10	10
	J. praehirsuta	*J. praehirsuta*	6	17	10	10
Inter-specific						
	J. albifrons	*J. praehirsuta*	10	10	10	10
	J. praehirsuta	*J. albifrons*	7	7	10	10

† Female from Brittany
‡ Female from Normandy

Table 2 - Components of reproductive isolation between marine isopods *Jaera albifrons* and *J. praehirsuta* in two regions with contrasting levels of interspecific gene flow. Reproductive isolation (RI) was calculated following Sobel & Chen (2014). Bootstrap confidence intervals (CI_{95}) based on 10000 resampling of observed data are given whenever sample sizes were not too small (e.g. sample sizes for survival of interspecific offspring in Brittany were particularly small since interspecific crosses were rarely successful). RI gives the limit to heterospecific gene flow that would be caused by each specific barrier acting alone. AC gives the actual absolute contribution of each barrier given that other barriers are acting earlier in life cycle. The sum of AC over all barriers is equal to the total strength of reproductive isolation. Sexual isolation contains a strong behavioural component, but could also include a (so far untested) gametic component. RI due to F1 hybrid inviability is based on brood size (number of offspring produced by a mother after intra-marsupial development) and survival of these offspring after 35 days.

Barrier	Notation	Parameter	Brittany				Normandy			
			Sample sizes		RI [CI_{95}]	AC	Sample sizes		RI [CI_{95}]	AC
Ecological isolation	RI_{eco}	Probability of heterospecific encounter	143 ind. under pebbles	79 ind. on algae	0.98 [0.955 ; 0.995]	0.977	101 ind. under pebbles	0 ind. on algae	0.00	0.00
Sexual isolation	RI_1	Proportion of successful crosses	21 intraspecific crosses	14 heterospecific crosses	0.73 [0.45 ; 1]	0.015	22 intraspecific crosses	15 heterospecific crosses	0.37 [0.06 ; 0.73]	0.37
F1 hybrid inviability	RI_2	Number of offspring released per brood	19 intraspecific broods	2 interspecific broods	0.06	0.001	16 intraspecific broods	5 heterospecific broods	-0.08	-0.07
	RI_3	Survival probability at 35 days	117 intraspecific offspring	11 interspecific offspring	-0.10	-0.001	148 intraspecific offspring	54 interspecific offspring	0.01 [-0.06 ; 0.09]	0.01
Total reproductive isolation	RI_{tot}				0.99	0.99			0.31	0.31

Figure 1

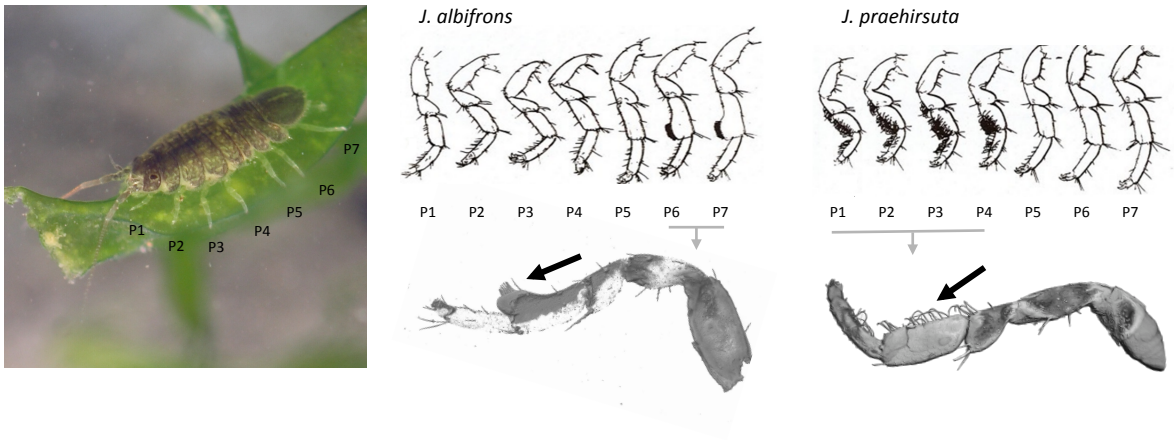

Figure 1

Sexual traits used for tactile courtship by males *Jaera albifrons* and *J. praehirsuta*. The second segment (carpus) of peraeopods 6 and 7 extend as a lobe bearing a patch of setae in males *J. albifrons*. Males *J. praehirsuta* instead have curved setae distributed on the first three segments (propus, carpus, merus) of peraeopods 1-4, and one or two spines on the carpus of peraeopods 6 and 7 Ribardière et al. (2017). The photography on the left shows a 4mm-long adult female (photo credit to Guillaume Evanno & Thomas Broquet). After fluorescent labelling of dissected appendices, close-up pictures were obtained with a confocal laser scanning microscope and processed using software Fiji and IMARIS.

Figure 2

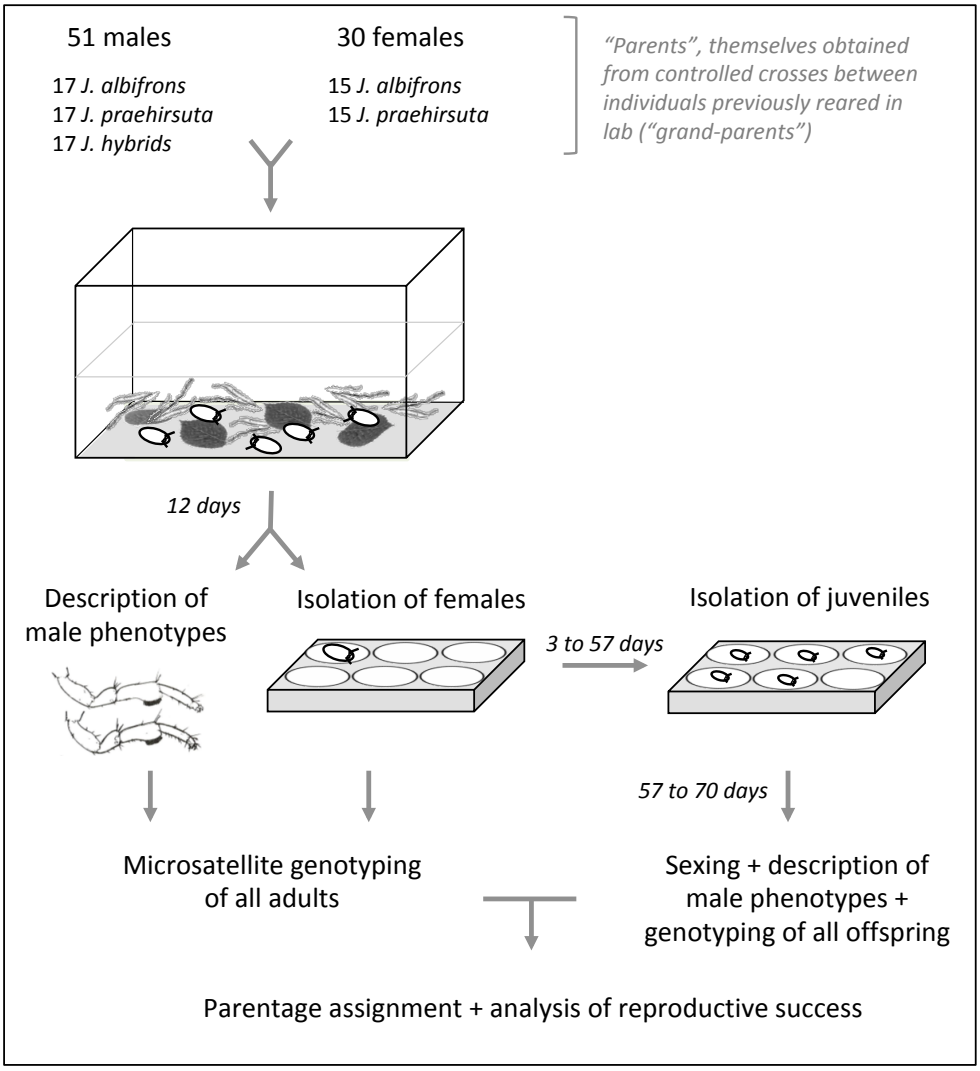

Figure 2

Protocol for the experimental "free-choice" population. Virgin adult males and females (that were themselves obtained from controlled crosses in the lab) were left in a small tank for 12 days during which they could freely interact. Females were then isolated in 6-well plates and surveyed until they released offspring. Each juvenile was then isolated in new 6-well plates and reared in the lab for ca. 60 days. The sexual traits of all males (both from the parental and offspring generations) were described. Finally, all individuals (potential mothers, potential fathers, and individuals from the new generation) were genotyped using 13 microsatellite loci and used in parentage analyses in order to assess the reproductive success of each adult.

Figure 3

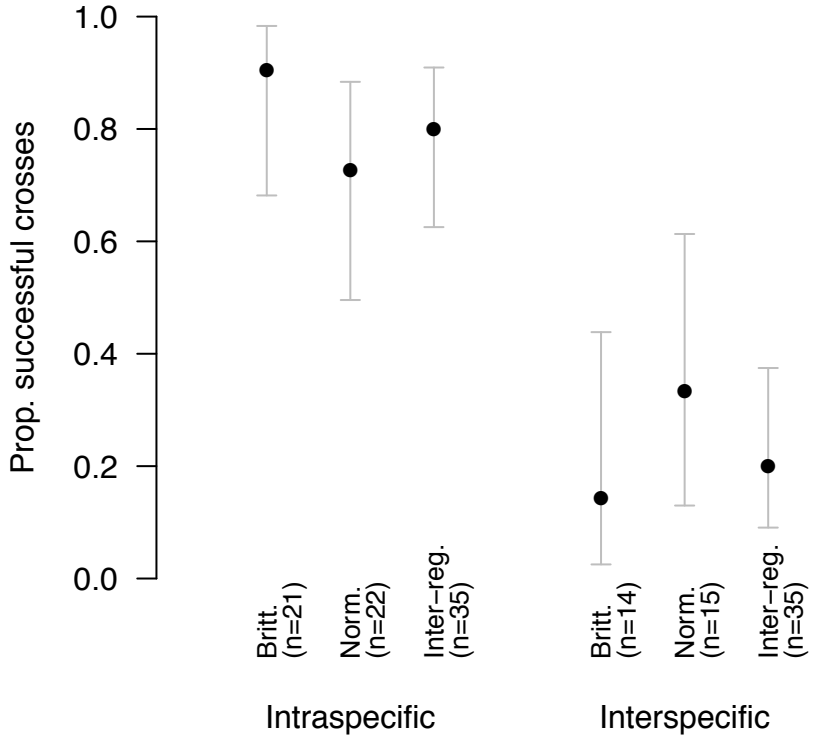

Figure 3

Proportion of successful crosses in no-choice experiments involving intra-specific crosses (either *Jaera albifrons* or *J. praehirsuta*) and inter-specific crosses. The male and female of a given cross could come from the same region (Brittany or Normandy, see text) or each from a different region (inter-reg. crosses). The sample sizes (number of experimental crosses) are given in brackets, and the bars give 95% confidence intervals around each observed proportion. A cross was successful if it produced at least one offspring. Interspecific crosses were consistently less successful than intraspecific ones, pointing towards sexual isolation between *J. albifrons* and *J. praehirsuta*.

Figure 4

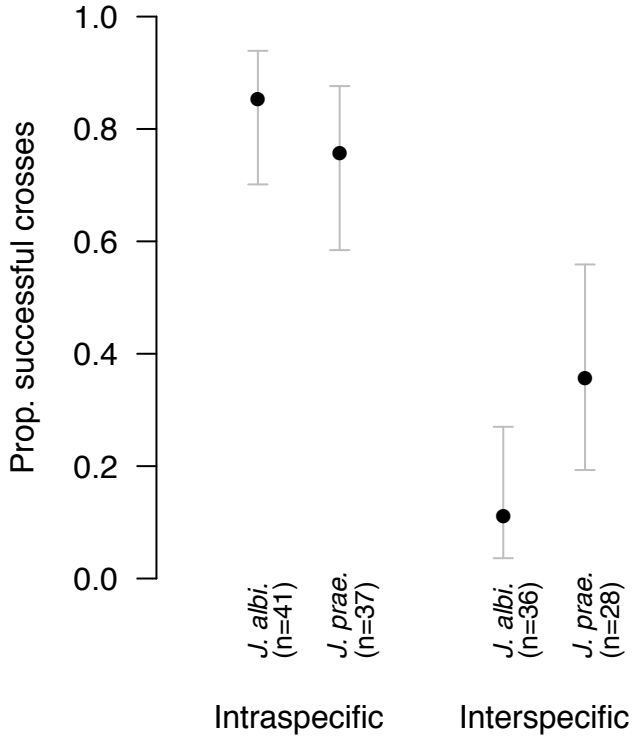

Figure 4

Proportion of successful intra-specific and inter-specific crosses in no-choice experiments involving either *J. albifrons* or *J. praehirsuta* females. The sample sizes (number of experimental crosses) are given in brackets, and the bars give 95% confidence intervals around each observed proportion. A cross was successful if it produced at least one offspring. Sexual isolation between species appears to be stronger in one direction (*J. albifrons* female).

Figure 5

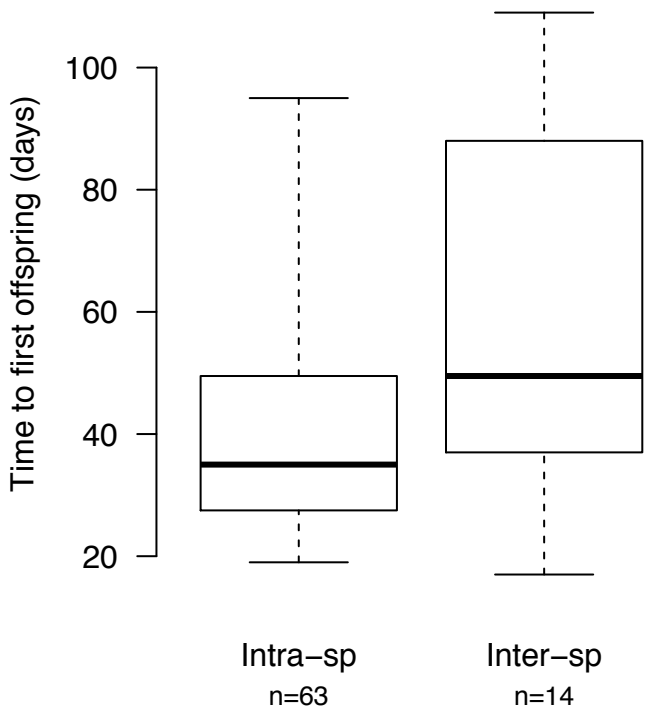

Figure 5

Delay between the start of each cross (when a virgin male and a virgin female are put together) and the release of the first brood. It takes longer for heterospecific pairs to produce offspring, indicating sexual isolation between species.

Figure 6

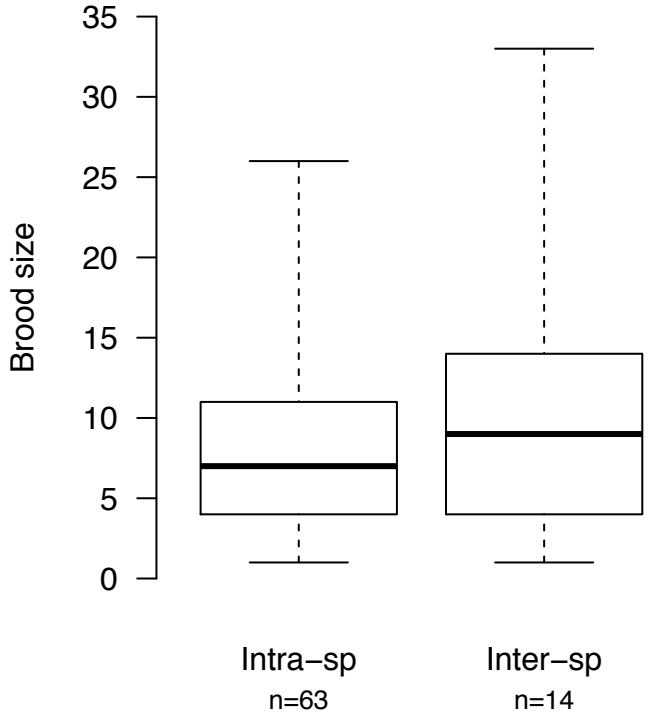

Figure 6

Number of offspring released per brood obtained from experimental no-choice intraspecific crosses (either *Jaera albifrons* or *J. praehirsuta*) and inter-specific crosses. The sample size (number of broods) is given below each box. There is no difference in brood size between cross types, indicating that no post-zygotic barrier is acting at this stage (embryo development in F1 hybrids).

Figure 7

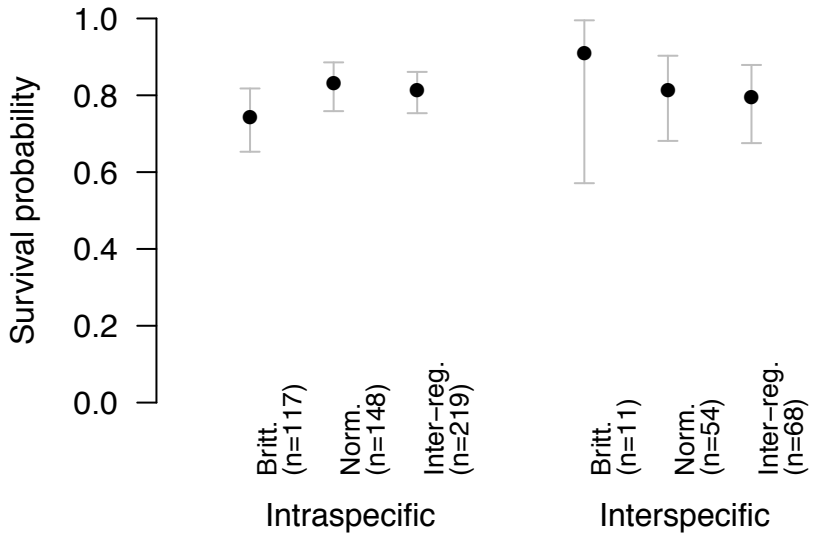

Figure 7

Proportion of offspring surviving at day 35. Offspring stem from experimental no-choice intraspecific crosses (either *Jaera albifrons* or *J. praehirsuta*) and inter-specific crosses. The male and female of a given cross could come from the same region (Brittany or Normandy, see text) or each from a different region (inter-reg. crosses). The sample sizes (number of experimental crosses) are given in brackets, and the bars give 95% confidence intervals around each observed proportion of offspring surviving.

Figure 8

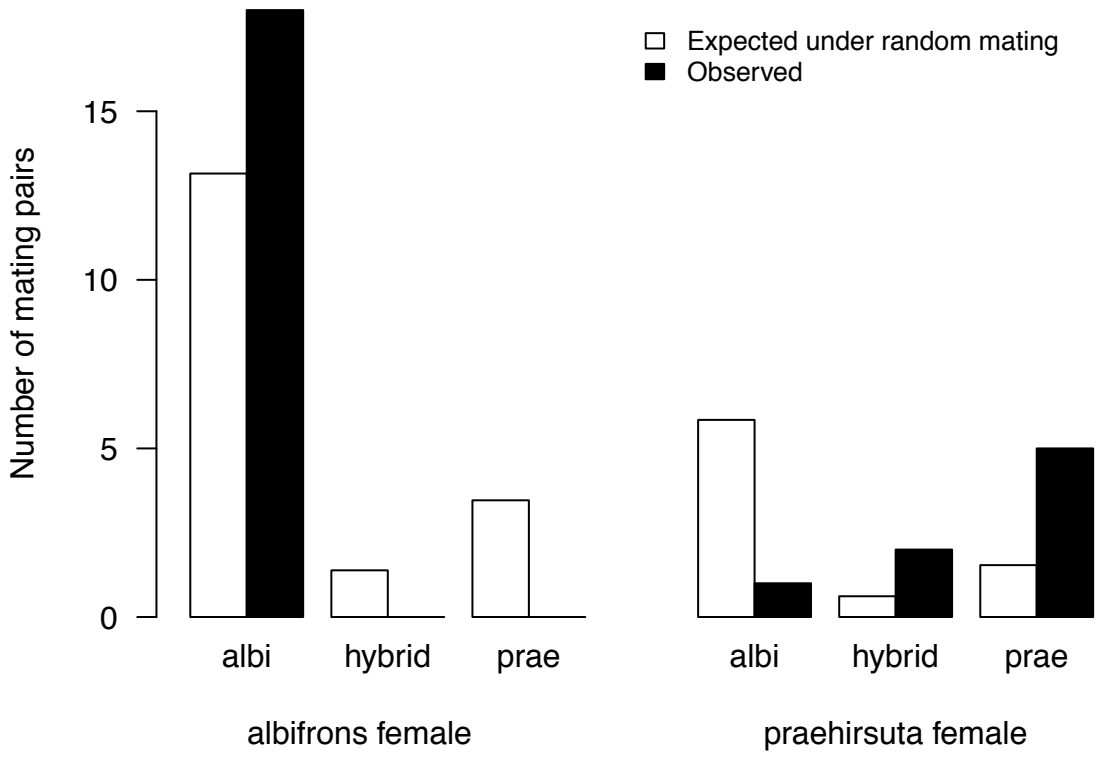

Figure 8

Number of mating pairs of each possible type expected from random mating in absence of sexual isolation (white bars) and observed from an experimental population (black bars). The expected numbers take into account not only the number of males and females of each type that entered the experiment but also their propensity to mate (see text). We see that *J. albifrons* females mated successfully only with *J. albifrons* males, while *J. praehirsuta* females produced offspring with males of the three different types, although not in equal proportions. These results point to strong but imperfect, asymmetrical sexual isolation.

Figure 9

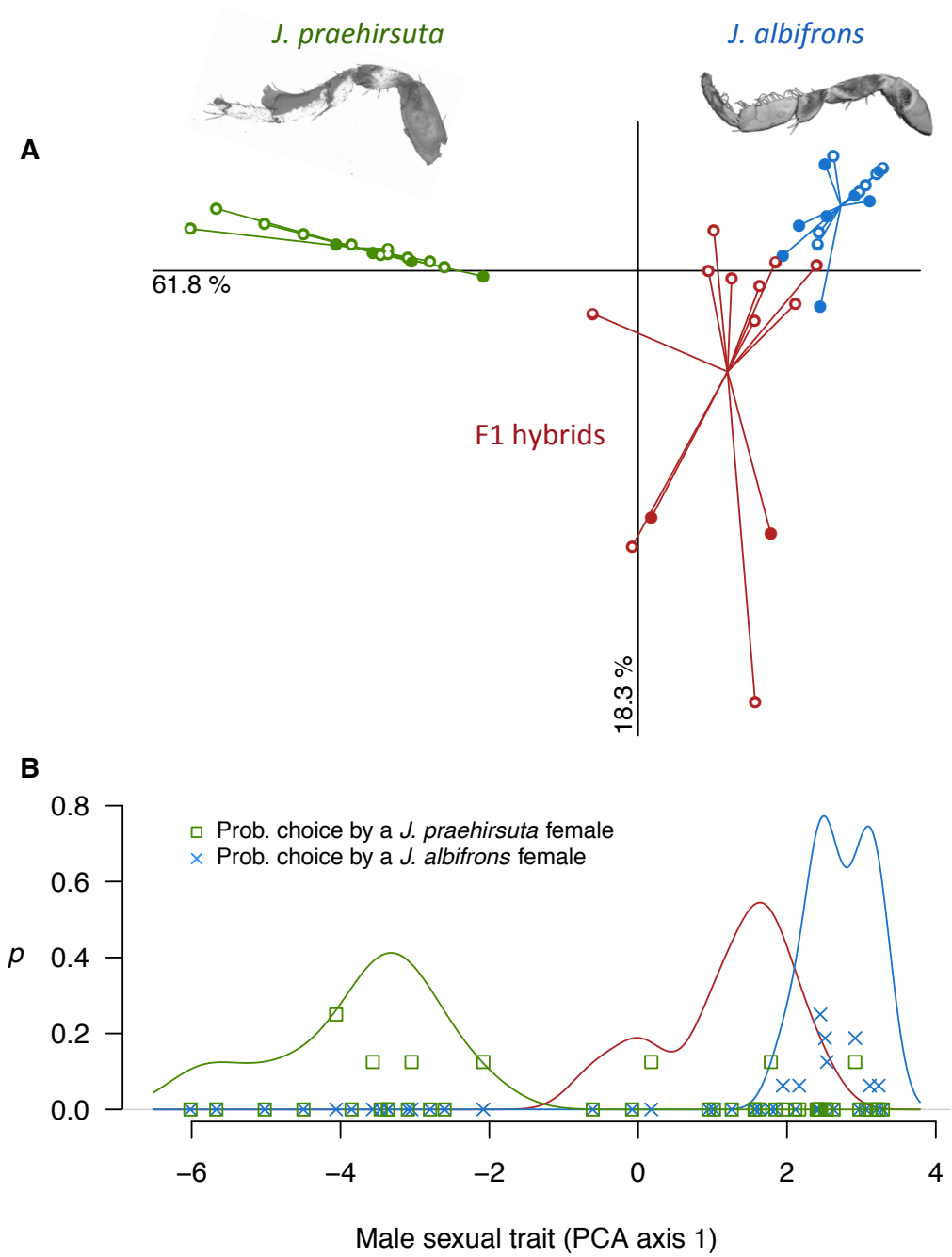

Figure 9

Asymmetric sexual isolation between *Jaera albifrons* and *J. praehirsuta*. Panel A) shows the first two components of a principal component analysis of male phenotypes (potential parents in the free-choice experimental population) grouped by types. We see that individuals of the *albifrons* or *praehirsuta* types are phenotypically differentiated, while individuals of the hybrid type (i.e.

produced by an "interspecific" cross) show more phenotypic variability, including phenotypes indistinguishable from the parental morphs. In this PCA plot, empty circles represent the males that did not sire any offspring, while solid dots show the males that successfully reproduced. In panel B), the solid curves show the density distribution of male sexual trait values along PCA axis 1 only. The probability that a female mated with a male showing a particular sexual trait value is shown by green squares (*J. praehirsuta* females) and blue crosses (*J. albifrons* females). The preferred mates of *J. albifrons* females coincide with the distribution of *J. albifrons* male sexual trait. By contrast, this concordance is much relaxed for *J. praehirsuta* females, which mated with males showing a wide range of trait values.