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# Geographically distinct patterns of reproductive isolation and hybridisation in two sympatric species of the *Jaera albifrons* complex (marine isopods)

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Running title: Isolation and hybridization in *J. albifrons*

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**Abstract**

Sympatric species that in some populations hybridize and in other populations remain reproductively isolated open interesting research possibilities for the study of hybridization and speciation. Here we test for such a situation in two littoral isopods (*Jaera albifrons* and *J. praehirsuta*) that occur in mixed populations and where past morphological descriptions suggested that the two species are generally reproductively isolated except in rare populations where hybridization may be happening. Using field surveys and microsatellite genetic structure analyses in two regions from France (Normandy and Brittany), we confirmed that introgressive hybridization occurs in a subset of mixed *J. albifrons* / *J. praehirsuta* populations (region Normandy) where the two species are found in the same habitat (pebbles on the shore). Moreover, we found that introgression in these populations is differential, 21 out of 23 microsatellite markers showing little genetic divergence between species (hierarchical analysis of molecular variance $F_{CT}$=0.017) while the remaining two loci were strongly differentiated ($F_{CT}$=0.428). By contrast, *J. albifrons* and *J. praehirsuta* in mixed populations from region Brittany occupied distinct habitats (pebbles and seaweeds, respectively) with little overlap and showed stronger genetic divergence ($F_{CT}$=0.132). In hybridizing populations, the majority of individuals show morphological traits that are characteristic of one or the other species. This raises the question of the forces that act to maintain this polymorphism, noting that hybridizing populations seem to be geographically isolated from potential source parental populations and show no detectable habitat divergence between species.

**Keywords**

Mosaic hybrid zone, isolating barriers, sexual isolation, genetic incompatibilities, introgression, crustaceans

## Introduction

Natural hybridization events inform our understanding of isolating barriers between species, the conditions of species coexistence despite hybridization, and the mechanisms of speciation. The archetypal hybrid zone structure is a region of contact between two otherwise allopatric species. In such hybrid zones, flanked on one side by populations of one species and on the other side by the other species, the dynamics of the system is most often driven by a balance between immigration from pure parental populations and selection against hybrids (the tension zone model, Barton & Hewitt, 1985). In such systems, individuals freely hybridize in the contact zone and hybrids have reduced fitness due to the segregation of genetic incompatibilities. Studies of naturally hybridizing populations have also increasingly highlighted the role of other isolating barriers, including environmentally-induced selection (Endler, 1977, Moore, 1977, Arnold, 1997) and sexual isolation (Seehausen et al., 1997, Poelstra et al., 2014).

Hybridizing populations vary not only in the nature of isolating mechanisms that are involved but also in geographic structure. Hybrid zones are typically characterized by a clinal structure (gradients of allelic frequencies between pure parental populations). More complex spatial structures are found when the environment induces differential selection on hybridizing species and the distribution of habitats is discrete (e.g. islands, lakes, host plants) or otherwise heterogeneous, leading to patchy hybrid zones (mosaic hybrid zones, Harrison & Rand, 1989, and other types of replicated hybridizing populations, reviewed e.g. in Harrison & Larson, 2016). Variable degrees of patchiness can also be induced by colonization history or population stochasticity (Gompert et al., 2010). Whatever causes patchiness, patchy systems allow us to compare multiple, potentially independent contact zones (McKinnon & Rundle, 2002, Bierne et al., 2003, Butlin et al., 2014). Such comparisons are also possible in hybrid zones that have a simpler spatial structure but that can be sampled along replicated transects (e.g. Teeter *et al*., 2010), and, notably, in experimental populations (Pritchard & Edmands, 2013). These comparative analyses may increase our

68 understanding of isolation mechanisms, their associated genomic architecture, and, promisingly,
69 speciation (Harrison & Larson, 2016, Westram et al., 2016).
70 Particularly intriguing are the situations where one can compare populations composed by a
71 mixture of individuals of two species that in some instances hybridize and in other instances remain
72 strongly reproductively isolated. That is, sympatric or mixed populations that may or may not be
73 reproductively isolated; hereafter we will use the term "mixed populations", defined as populations
74 where individuals of two species are close enough so that they can meet and interact frequently. An
75 illustrative case in point is the lake Victoria cichlid 'speciation transect' (Seehausen, 2009) where
76 mixed populations of *Pundamilia pundamilia* and *P. nyererei* show more or less hybridization
77 depending on variations in premating behavioral mechanisms themselves linked with variations in
78 habitat (water clarity). Fish studies have provided a few other related examples where a pair of
79 species shows contrasted levels of reproductive isolation when in sympatry (benthic and limnetic
80 three-spined stricklebacks, Taylor et al., 2006, swordtail fish, Culumber et al., 2011, and lake
81 whitefish, Gagnaire et al., 2013, river and blueback herring, Hasselman et al., 2014, and river and
82 brook lampreys, Rougemont et al., 2015). Comparing sympatric non-hybridizing / hybridizing
83 populations provides power to interpret admixture patterns (e.g. shared ancestral polymorphism *vs*
84 current gene flow) or assess whether differential introgression patterns are due to heterogeneous
85 recombination, selection, or gene flow (Gagnaire et al., 2013, Powell et al., 2013, Rougemont et al.,
86 2016).
87 Here we focus on the *Jaera albifrons* group, a complex of five marine isopod species that live
88 on the shores of the temperate and cold waters of the North-Atlantic Ocean. It includes *J. albifrons*, *J.*
89 *praehirsuta*, *J. ischiosetosa*, *J. forsmani*, and *J. posthirsuta* (Bocquet, 1953, Naylor & Haahtela, 1966,
90 Bocquet, 1972). Note that *Jaera albifrons* designates one of the five species of the *Jaera albifrons*
91 group (the distinction will be noted using the words "complex" or "group" throughout). All five
92 species occupy a narrow but geographically extended belt in the intertidal zone and they have largely
93 overlapping distribution ranges. In short, individuals from one species frequently coexist with

94 individuals from at least one other species throughout their distribution range, and mixed
95 populations are the rule rather than the exception. In this context, the five species of the *Jaera*
96 *albifrons* group were shown to be reproductively isolated by at least three types of barriers: i)
97 ecological isolation (variations in local habitat preferences along the seashore), ii) sexual isolation
98 (differences in male secondary sexual traits used in tactile courtship, and strong female-driven mate
99 choice), and iii) genetic incompatibilities (reviewed in Solignac, 1978, 1981, Mifsud, 2011). The
100 reproductive isolation resulting from the combination of these pre- and post-zygotic barriers is
101 thought to be very strong in nature.
102 However, intermediate male sexual traits have been reported in a few populations, suggesting
103 that hybridization may happen in some rare places (Solignac, 1978). One such potentially hybridizing
104 population has been intensively studied by Charles Bocquet and Michel Solignac between 1965 and
105 1970. They described a *Jaera albifrons* / *J. praehirsuta* mixed population located in Luc-sur-Mer,
106 Normandy (France) where the analysis of male secondary sexual traits and experimental crosses led
107 them to conclude that this population contained an exceptional proportion of hybrids (15 to 32%
108 depending on sampling event and classification thresholds, Bocquet & Solignac, 1969, Solignac,
109 1969a, b, 1978). Based on morphological descriptions for a large number of individuals sampled or
110 raised in the lab from this population (nearly 2000 ind., Solignac, 1978) and comparison with
111 experimental crosses (Bocquet & Solignac, 1969), their conclusion on hybridization between species
112 seems very strong. This past work convincingly suggests that hybridization was occurring in at least
113 one particular site in 1965-70 while all other known *J. albifrons* / *J. praehirsuta* sympatric populations
114 were reproductively isolated. As discussed above, such a situation seems interesting for the study of
115 isolating barriers and speciation.
116 With this study, our objectives were i) to test for hybridization between *J. albifrons* and *J.*
117 *praehirsuta* using genetic tools, ii) to investigate the geographic structure of hybridizing populations
118 and the nature of the isolating mechanisms, and iii) to compare genetic patterns within hybridizing *vs*
119 non-hybridizing mixed populations. For this purpose, we searched for mixed populations and

morphologically intermediate individuals as described in Normandy ca. 50 years ago, analyzed the genetic structure of local populations using a panel of 23 microsatellite loci, and compared it with mixed populations from another French region (Brittany) where the two species had been described as reproductively isolated.

## Materials and methods

### Model species

Members of the *Jaera albifrons* complex are small marine crustaceans (2-5 mm total adult length, Fig. 1). Out of the five species comprising the *Jaera albifrons* complex, two are restricted to the temperate waters of the North-American east coast (*J. posthirsuta*) or the European coast (*J. forsmani*), while the three others are more widely distributed on both sides of the Northern Atlantic (Bocquet, 1972). These five species are found in abundance in the intertidal zone, where they can show local habitat preferences involving variations in micro-habitat (under rocks or on seaweeds), level on the intertidal zone, and salinity (Naylor & Haahtela, 1966, Jones, 1972). However, these habitat preferences vary widely, meaning that ecological isolation is also very variable (Veuille, 1976, Solignac, 1981).

The identification of species within the *Jaera albifrons* complex is based on male secondary sexual traits (Fig. 1). Mating is preceded by a courtship behavior whereby males mount females in a head-to-tail position and used different parts of their peraeopods to brush or press the female's back. Males of the five species differ in the distribution of setae and spines borne by the peraeopods used to court females (Jones & Fordy, 1971, Solignac, 1978), and a female's acceptance or rejection is a major driver of reproductive isolation between species.

### Species survey and sampling

We sampled *J. albifrons* and *J. praehirsuta* in two regions. First we focused on the area where Michel Solignac had described hybridization between these two species in 1965 and 1970 (Solignac,

1978). For that, we surveyed strictly all potential habitats on a 25 Km portion of the coast around this original site, finding the population studied by M. Solignac to be extinct (Luc-sur-Mer, Fig. 2), possibly due to the regular removal of pebbles from the beach for touristic activities. We extended this survey 35 Km East and 35 Km West by visiting a large number of (but not strictly all) potential habitats, where we found three sites with a mixture of *J. albifrons* and *J. praehirsuta* (sites 7-9, see Results, Table 1, and Fig. 2). This gave us a 95 Km continuous portion of the coastline where we have a precise, although not striclty exhaustive, view of the distribution of species (from Grandcamp-Maisy to Honfleur, second world war landing beaches, highlighted in yellow in Fig. 2).

Less intensive surveys were more recently conducted further West and East in order to check for additional mixed or pure *J. albifrons* / *J. praehirsuta* populations (such populations were found but not analyzed in this study, see Results).

Second, we searched for similarly mixed populations of the same pair of species in a region where no hybridization had been found despite extensive field studies (area around Roscoff biology station, Brittany, France, Fig. 2, Bocquet & Solignac, 1969, Solignac, 1969b). In this region we selected 6 sampling sites where the two species were found at the same location (Fig. 2 and Table 1).

In both regions (Brittany and Normandy) we looked for individuals of the *Jaera albifrons* complex under rocks and on seaweeds (*Ascophyllum nodosum*, *Fucus vesiculosus* and *Fucus serratus* essentially) in the intertidal zone. Animals found on rocks were collected in the field using a small brush. By contrast, samples of seaweeds were brought back to the lab where we checked for the presence of *Jaera* individuals by shaking algae repeatedly in freshwater (Solignac, 1978). All individuals where kept alive until identification based upon observation of male secondary sexual traits. Because females of all species are morphologically identical, this study is based on male individuals only. As adult females are larger than males, many females could be left alive in the field. All collected individuals were fixed in ethanol after species identification.

Finally, one sampling site (Ste-Honorine-des-Pertes, site 8 in figure 2) was selected for a detailed analysis of the micro-distribution of individuals. In this site we performed an exhaustive

172 survey on a large portion of the beach, recording the precise localization of each individual with a
173 Trimble GeoExplorer 6000 GPS (average horizontal accuracy 55 mm). There we also collected
174 females, which were kept in the lab until they produced offspring (sperm storage allows females to
175 produce offspring in absence of males). These offspring were reared in the lab for at least six weeks,
176 until each individual could be sexed and each male could be identified based on secondary sexual
177 traits. This identification of series of male sibs gave a presumptive species identification for their
178 mother (e.g. a female could be classified as *J. albifrons, J. praehirsuta* or "hybrid" if it gave birth to a
179 series of males bearing *J. albifrons*, *J. praehirsuta* or intermediate morphological traits, respectively).
180
181 Genetic analyses
182 All genetic analyses are based on individual genotypes obtained at 23 microsatellite loci (all
183 loci described in Ribardière et al., 2015, except Ja01). DNA extraction and amplification followed the
184 protocols described in Ribardière *et al*. (2015). Pairwise linkage disequilibrium between loci was
185 tested in FSTAT version 2.9.3.2 (Goudet, 2001) in order to avoid redundant information. Departure
186 from Hardy-Weinberg equilibrium was also tested in FSTAT in order to detect technical artifacts (null
187 alleles or allelic dropouts) or departure from random mating within sampling sites. The occurrence of
188 null alleles, already detected in the *Jaera albifrons* complex with these microsatellites (Ribardière *et*
189 *al*., 2015) was specifically investigated with the software MICROCHECKER (Van Oosterhout *et al*., 2004).
190 About 10% of the genotypes where randomly replicated in order to evaluate the frequency of
191 genotyping errors and for each locus genotyping error rate was calculated as error rate = (number of
192 false genotypes) / (total number of repeated genotypes). The level of polymorphism was estimated
193 by measuring observed and expected heterozygosity in FSTAT.
194 Our test of reproductive isolation or hybridization between *J. albifrons* and *J. praehirsuta*
195 within our two sampling regions (Brittany and Normandy) is based upon estimates of genetic
196 differentiation. We estimated the distribution of genetic variance among sampling sites within a
197 species ($F_{SC}$) and between species ($F_{CT}$) in a hierarchical analysis of molecular variance (AMOVA,

198 Excoffier et al., 1992) implemented in ARLEQUIN version 3.5 (Excoffier & Lischer, 2010). We performed
199 this analysis independently within each region. The between-species component $F_{CT}$ will thus inform
200 us on the strength of genetic differentiation between *J. albifrons* and *J. praehirsuta* within each
201 region. Small samples were not included in these analyses (that is, individuals with an intermediate
202 phenotype, and individuals from site 9, where one of the two species was represented by only one
203 individual). These analyses were performed using both allelic frequencies ($F_{ST}$-like) and the distance
204 between alleles ($R_{ST}$-like) and significance was assessed using 10100 permutations as implemented in
205 ARLEQUIN. We ran these analyses first using all markers (global AMOVA) and then for each
206 microsatellite locus independently (locus-by-locus AMOVA).
207 Because some loci showed strongly contrasted levels of between-species genetic structure
208 ($F_{CT}$) in Normandy *vs* Brittany (see results), the significance of the difference between $F_{CT\text{-Normandy}}$
209 against $F_{CT\text{-Brittany}}$ was tested by bootstrapping individuals 1000 times in R version 3.2.3 (R Core Team,
210 2016). This allowed us to estimate how often the two $F_{CT}$ values obtained from a given resampled
211 dataset overlapped, and thus whether $F_{CT\text{-Normandy}}$ differed significantly from $F_{CT\text{-Brittany}}$ at the locus
212 tested.
213 Pairwise estimates of genetic differentiation between samples were also obtained in a non-
214 hierarchical model in ARLEQUIN (that is, $F_{ST}$ between all pairs of populations, where a population is
215 defined by a given species in a given sampling site, Table 1). This is useful i) to evaluate whether the
216 global differentiation between species is consistent across sampling sites (i.e. using $F_{ST}$ between
217 species within each site separately), and ii) to investigate genetic structure within each species
218 separately, in particular by testing for isolation by distance between sampling sites with SPAGEDI 1.4
219 (Hardy & Vekemans, 2002) and GENEPOP 4.2.2 (Rousset, 2008).
220 Finally, the distribution of genetic variance was also investigated using individual analyses
221 without a priori grouping of samples. We first ran a clustering analysis within each region using
222 STRUCTURE 2.3.4 (Pritchard *et al*., 2000) with an admixture model (10 independent repetitions, burn-in
223 period= 50000, MCMC= 300000). The most likely number of clusters (*K*) was determined *via*

224 HARVESTER v.0.6.1 (Earl, 2012) using Δ*K* as described by Evanno *et al*. (2005). The *Ancestdist* option
225 was used in STRUCTURE to calculate 95% probability intervals for an individual's membership *q* to
226 each cluster. The width of such intervals (difference between upper and lower bounds) gave us an
227 estimate of the precision of membership values. Second, we performed a Principal Component
228 Analysis (PCA) based on all individual genotypes using the R package ade4 (Dray & Dufour, 2007).
229 Because the locus-by-locus AMOVA showed that two loci had a striking behavior (see results), we ran
230 both analyses (PCA and STRUCTURE) with and without these two loci.
231
232 **Results**
233 **Population survey**
234 In Brittany we sampled 142 *J. albifrons* and 80 *J. praehirsuta* in six sites where the two species
235 were co-occurring (Fig. 2 and Table 1, only males are considered throughout the paper, unless stated
236 otherwise). In these sites, all *J. albifrons* but two were found under rocks, while all *J. praehirsuta*
237 except three were found on seaweeds (located directly above rocks or within a radius of a few
238 meters). In this region, all individuals could be morphologically identified at the species level without
239 any overlap of traits (i.e. no individuals with intermediate morphology).
240 In Normandy we sampled 44 *J. albifrons* and 57 *J. praehirsuta* from the three mixed
241 populations that we found (sites 7-9, Fig. 2). Contrary to the Brittany situation, all individuals were
242 collected under rocks, while no individuals could be found on seaweeds. In addition, we found 9
243 individuals clearly showing intermediate morphological traits as described by Solignac (Fig. 1, "hybrid
244 types" 5 to 13 in Solignac 1978, p.172-177). These individuals will be referred to as "intermediate
245 phenotypes" hereafter. The portion of the coast that was intensively surveyed revealed a single pure
246 *J. albifrons* population located >70 Km East of mixed populations (empty circle in figure 2).
247 Later, less intensive surveys revealed another pure *J. albifrons* population 30 Km West (Fig. 2),
248 and interestingly, one additional mixed *J. albifrons* / *J. praehirsuta* population (with intermediate

249 phenotypes) further East (location Yport, Fig. 2). No *J. praehirsuta* individuals were found outside of
250 mixed populations anywhere in Normandy.
251 The fine-scale distribution of individuals at site 8 (Ste-Honorine-des-Pertes, Normandy)
252 showed that individuals (61 males and 138 females) of the two species and intermediate phenotypes
253 were largely intermingled, with *J. praehirsuta* being distributed all along the shore while the
254 distribution of *J. albifrons* was more irregular (supplementary material Fig. S1).
255
256 **Genetic diversity**
257 All microsatellite loci could be amplified in individuals of the two species (consistent with
258 Ribardière *et al*., 2015) as well as in individuals with intermediate phenotypes. There was no linkage
259 disequilibrium after Bonferroni correction between all pairs of loci in each population, so that all 23
260 markers were kept for further analyses. The level of polymorphism was globally consistent across
261 species (Table 1). Unless stated otherwise, individuals with intermediate phenotypes were removed
262 from the following analyses for we had too few of them (2 in Grandcamp, site 7, and 7 in Ste-
263 Honorine-des-Pertes, site 8, Fig. 2). Observed heterozygosity *H*o (0.45 in *J. albifrons* and 0.51 in *J.*
264 *praehirsuta*) was on average lower than within population gene diversity *He* (0.51 and 0.59,
265 respectively), resulting in a significant departure from Hardy-Weinberg equilibrium ($F_{IS}$ values in
266 Table 1). Departure from HWE was driven in Brittany by loci Ja37, Ja39, Ja55 and Ja94, and in
267 Normandy by locus Ja55 (loci with significant positive $F_{IS}$ in one to six samples, data not shown).
268 These loci, except Ja94, showed signs of a null allele in more than half of populations, as tested using
269 MICROCHECKER. While we did not detect locus-specific HW disequilibrium patterns that were
270 consistent across populations, the main downstream quantitative analysis (analysis of molecular
271 variance, see below) was run with and without the four loci cited above (and we report locus-specific
272 results as well). Genotyping error rates estimated from replicated individuals ranged from 0 to 5.88%
273 per locus (average 1.7%), and were due roughly equally to allelic dropouts and false alleles.
274

**Genetic differentiation between *J. albifrons* and *J. praehirsuta***

The hierarchical analysis of molecular variance based on 23 microsatellite loci (Table 2) showed that the between-species differentiation was higher in Brittany ($F_{CT\text{-}Brittany}$ = 0.132, $p$ < 0.005) than in Normandy ($F_{CT\text{-}Normandy}$ = 0.074, $p$ = 0.34). Accordingly, the locus-by-locus AMOVA showed that most loci were less differentiated between species in Normandy than in Brittany (Fig. 3). However, two loci (Ja41 and Ja64) revealed a strikingly different pattern: these two loci showed a very strong level of differentiation (Ja41, $F_{CT\text{-}Normandy}$ = 0.462; Ja64, $F_{CT\text{-}Normandy}$ = 0.384) in Normandy region while the remaining 21 loci showed no or little differentiation (locus specific $F_{CT\text{-}Normandy}$ ranged from -0.05 to 0.079], Fig. 3). Without these two peculiar loci, there is thus no genetic differentiation between species in Normandy ($F_{CT\text{-}Normandy}$ = 0.017, $F_{CT\text{-}Brittany}$ = 0.125, table 2). Note that this result is unchanged when removing the four loci that showed a departure from HW equilibrium in some populations (not shown).

Moreover, the differentiation between species at loci Ja41 and Ja64 in Normandy was not only much stronger than at other loci but it was also stronger than the differentiation observed at the same two loci in Brittany (Ja41, $F_{CT\text{-}Brittany}$ = 0.199, Ja64, $F_{CT\text{-}Brittany}$ = 0.194, Fig. 3), and this difference was significantly different from 0 (bootstrap $p$-value<0.001 for Ja41 and $p$=0.001 for Ja64, supplementary material Figs. S2 and S3). The distribution of allelic frequencies at these two loci is presented in supplementary material figures S4 and S5.

Although the heterogeneity across loci appeared somewhat lower in Brittany (Fig. 3), there was nonetheless some variation, with two other loci showing a particularly strong level of differentiation (Ja66, $F_{CT\text{-}Brittany}$ = 0.605; Ja80, $F_{CT\text{-}Brittany}$ = 0.37). The difference in $F_{CT}$ between regions was significant at these two loci (bootstrap $p$-value<0.001 for Ja66 and Ja80, supplementary material figure S3).

Because we studied only three mixed populations in Normandy, and one of them contained nearly only *J. albifrons* (site 9, Longues-sur-Mer, Fig. 2, Table 1), the permutation procedure implemented in ARLEQUIN to test for the significance of $F_{CT\text{-}Normandy}$ is essentially powerless (the

between-species component of genetic variation is tested by permuting populations within species). We therefore checked if the patterns found in the AMOVA (which considers all sites simultaneously) were consistent across sites. Tables 3 and 4 show pairwise $F_{ST}$ values calculated in a simple non-hierarchical framework. Most importantly, it shows that the between-species differentiation was consistent across sites, both in Normandy ($F_{ST}$ between species equal to 0.104 at Grandcamp, site7, and 0.101 at Ste-Honorine-des-Pertes, site 8) and in Brittany ($F_{ST}$ between species within sites in [0.1; 0.19]). These results consider all loci, but the same geographical consistency is observed when considering the locus-specific patterns described above (data not shown). That is, the global AMOVA results are repeatable across sites (e.g. $F_{ST}$ at the two sites from Normandy = 0.546 (site 7) and 0.396 (site 8) when considering only loci Ja41 and Ja64, and $F_{ST}$ = 0.05 (site 7) and 0.056 (site 8) with all other loci).

Individual analyses bring some complementary information, in particular because the individuals with intermediate morphology could be included in spite of their low abundance (as well as individuals from site 9). Running STRUCTURE with K=2, we found that *J. albifrons* and *J. praehirsuta* cluster into two clearly identified groups both in Brittany and Normandy using a panel of 23 loci (Fig. 4). However, while an individual's membership *q* to its assigned cluster was similar for both species in Brittany and Normandy (membership averaged over all individuals and 10 STRUCTURE runs, Brittany: $\overline{q}_{albifrons}$ = 0.99, $\overline{q}_{praehirsuta}$ = 0.97, and Normandy: $\overline{q}_{albifrons}$ = 0.97, $\overline{q}_{praehirsuta}$ = 0.95), the uncertainty associated with *q* was larger in Normandy (average width of 95% probability interval $w^{95}_{albifrons}$ = 0.24, $w^{95}_{praehirsuta}$ = 0.32) than Brittany ($w^{95}_{albifrons}$ = 0.12, $w^{95}_{praehirsuta}$ = 0.19). As it turned out, the apparent genetic clustering in Normandy was almost entirely due to the effect of two loci only (Ja41 and Ja64), while a STRUCTURE analysis using the remaining 21 loci showed that the two species were genetically homogeneous (Fig. 4B).

The results from molecular analyses of variance and clustering analyses could be well visualized using PCA performed with all 23 loci (Figure 5). The two isopod species were clearly differentiated in one region (Brittany) and less so in the other one (Normandy). We see also that

individuals with intermediate phenotypes were genetically indistinguishable from individuals with *J. praehirsuta* traits.

The particular geographical distribution of individuals (replicates of mixed populations comprising intermediate phenotypes and absence of clinal structure, see discussion) precluded the use of genetic tools dedicated to the analysis of hybridization in clinal hybrid zones.

**Genetic structure within species**

Because there is ongoing hybridization and introgression between the two species in Normandy (see discussion), the within-species genetic structure is best investigated using samples from Brittany. Brittany is also the region where we have more sampling locations. The genetic structure among *J. albifrons* samples appeared slightly higher than within *J. praehirsuta*. This is visible from pairwise $F_{ST}$ estimates (Table 3), ranging from 0.005 to 0.066 (13 out of 15 pairs significantly different) when considering only *J. albifrons* within Brittany, and -0.075 to 0.017 (1 out of 15 pairs significant) when considering only *J. praehirsuta*.

In addition, a significant pattern of isolation-by-distance (sup. Fig. S6) was observed in Brittany both in *J. albifrons* ($R^2 = 0.73$, Mantel test *p*-value < 0.01) and *J. praehirsuta* ($R^2 = 0.11$, *p*-value = 0.02). In line with the pairwise $F_{ST}$ results, genetic differentiation increased more rapidly with distance in *J. albifrons* than in *J. praehirsuta*, although 95% confidence intervals calculated in GENEPOP overlapped (10000 permutations, *J. albifrons* [0.013, 0.037], *J. praehirsuta* [-0.0005, 0.018], Fig. S6).

**Discussion**

The first result of this study is the clear-cut confirmation that introgressive hybridization is happening between *J. albifrons* and *J. praehirsuta* in at least two mixed populations from Normandy, France. As developed below, this opens interesting questions regarding the conditions of coexistence of the two parental morphs in hybridizing populations that seem to receive no influx from pure parental populations and shows no detectable ecological heterogeneity.

1- Hybridization between *Jaera albifrons* and *J. praehirsuta*

Analyses of molecular variance (Table 2 and Fig. 3) and admixture analyses (Figs. 4 and 5) both showed that mixed populations from Normandy have a homogeneous genetic structure at 21 out of 23 multi-allelic loci. Critically, this genetic homogeneity contrasts with the differentiation observed in mixed populations from Brittany, where individuals bearing sexual traits specific to *J. albifrons* or *J. praehirsuta* cluster into clearly marked genetic groups (Figs. 4A and 5). Hence shared ancestral polymorphism cannot explain the lack of differentiation between species in Normandy, which therefore supports the hypothesis of ongoing hybridization.

These findings agree with the conclusions reached by C. Bocquet and M. Solignac nearly 50 years ago, who studied the morphological variation of secondary sexual traits in a population from the same region (Luc-sur-Mer, Fig. 2, Bocquet & Solignac, 1969, Solignac, 1969a, b, 1978 chapter 6). Similarly to the results reported by these authors, we found that in Normandy several males have intermediate phenotypes and the two species occupy the same habitat (under stones and pebbles on the shore) while in Brittany we did not detect any intermediate phenotypes and the two species occupy two different habitats, with some overlap; *J. albifrons* lives primarily under stones, while *J. praehirsuta* is found primarily on seaweeds.

We conclude from these observations and the contrast in species divergence in the two regions that the two species are currently hybridizing in populations from Normandy, but not in Brittany.

2- A semi-permeable barrier to gene flow

The genetic homogeneity observed across species in Normandy further shows that hybridization has been introgressive, as correctly concluded by Solignac (1969b) from the continuous range of morphological characteristics observed in natural populations and by comparison with experimental crosses (Bocquet & Solignac, 1969). While it is now clearly established that

379 introgression proceeds differentially across loci in hybrid zones (with no known exceptions, Harrison
380 & Larson, 2014), investigating this variation was not part of our original plan with this study given
381 that we were using a panel of only 23 loci. However, the locus-by-locus AMOVA analyses revealed a
382 surprisingly trenchant pattern, whereby 21 loci showed no differentiation at all between species in
383 populations from Normandy ($F_{CT}$ in [-0.05; 0.08]) and the two remaining loci where strongly
384 differentiated ($F_{CT}$ = 0.384 and 0.462). Moreover, these two loci were also significantly more
385 differentiated in the hybridizing populations (Normandy) than in reproductively isolated ones
386 (Brittany) while showing no differentiation within each species (see $F_{SC}$ values in Table 2). This
387 strongly suggests that there is a semi-permeable barrier to gene flow between *J. albifrons* and *J.*
388 *praehirsuta* in hybridizing populations from Normandy. Hypotheses other than a reduction in gene
389 flow at these loci seem impossible to reconcile with the fact that the same two loci are significantly
390 less differentiated in non-hybridizing sympatric populations. Alternate hypotheses such as the
391 differential sorting of ancestral polymorphism or reduced variability at these loci due to a locally low
392 recombination rate would require a history of differentiation between species whereby ancestral
393 polymorphism or recombination have evolved differentially in the two regions studied (ca 250km
394 apart). While this is theoretically possible, a more parsimonious hypothesis is that the two loci are
395 encompassed in one or two genomic regions where inter-specific gene flow is hampered because
396 these regions are linked with one or several isolating barriers.
397 Additional indicators of a semi-permeable barrier to gene flow are two other markers (Ja66
398 and Ja80) also showing a heterogeneous pattern. As most markers they are more differentiated in
399 Brittany than in Normandy, but, interestingly, they show a stronger differentiation than the other loci
400 in Brittany (Fig. 3). We cannot currently make assumptions based only on these results, which
401 emphasize the necessity to study the heterogeneity of genome with an extended set of genetic
402 markers.
403
404 3- Is introgression symmetrical?

405 Interspecific crosses are generally not equally likely in both directions, especially when
406 behavioral isolation is involved (e.g. Coyne & Orr, 2004, p. 226). Such asymmetries leave specific
407 signatures in the genome that are most easily detected by comparing uni- and bi-parentally inherited
408 genetic variation (typically, markers from the mitochondrial and nuclear DNA, e.g. Toews & Brelsford,
409 2012). It would be interesting to test for asymmetric introgression of mtDNA in our system given that
410 Bocquet and Solignac (1969) have suggested that interspecific crosses may occur more easily in one
411 direction (female *J. praehirsuta* x male *J. albifrons*) than the other. This result was obtained from
412 experimental crosses with individuals from a hybridizing population (Luc-sur-Mer, Normandy), and
413 the asymmetry was further confirmed by Solignac (1981) using individuals from other origins (non-
414 hybridizing populations). Yet we could not use this approach here because mtDNA analyses so far
415 have indicated that most of the genetic variation is shared by all species of the *Jaera albifrons*
416 complex (perhaps excluding the American species *J. posthirsuta*, which has not been included in
417 these analyses). That is, the four European species form a polyphyletic clade (16S rDNA, Mifsud,
418 2011, and COI, Ribardière and Broquet unpublished), in strong contrast with the patterns obtained
419 with nuclear data (AFLP, Mifsud, 2011, and microsatellites, this study). There are no mitochondrial
420 haplotypes or clades that are specific to *J. albifrons* or *J. praehirsuta* (not shown), and the cyto-
421 nuclear discordance, also certainly interesting in its own right, is not informative of recent
422 introgression directionality. The symmetry of introgression can in some cases be evaluated by taking
423 advantage of differences between nuclear loci (differential introgression), but our microsatellite
424 dataset is too limited for this approach. Nevertheless, results of admixture analyses show that
425 individuals showing an intermediate phenotype share more genetic background with *J. praehirsuta*
426 (Figs. 4 and 5) which suggests that introgression is asymmetric (genetic variation from *J. albifrons*
427 introgressing into the genetic background of *J. praehirsuta*). This can also be seen by looking at allelic
428 frequencies at loci Ja41 and Ja64 for the phenotypically intermediate individuals, which are similar to
429 that of individuals bearing *J. praehirsuta* traits and different from the frequencies observed in *J.*
430 *albifrons* (supplementary material Figs. S4 and S5).

4- Geographical structure and persistence of hybridizing populations

In a 1969 paper, C. Bocquet and M. Solignac reported that many "morphological hybrids" have been observed during the preceding 15 years in their study area of Luc-Sur-Mer (Bocquet & Solignac, 1969). There is no more suitable habitat at this site, but Solignac (1978, p. 171) mentioned that "hybrids" had been found in Ste-Honorine-des-Pertes, which is one of the two sites sampled in the present study (site 8). This means that hybridizing populations have persisted in Normandy for at least several decades. Moreover, during a recent additional survey aiming at extending the 95 Km coastline region studied here, we found intermediate phenotypes in a population located roughly 100 Km East of the mixed populations studied here (location "Yport", Fig. 2). Even more unexpectedly, we detected a *J. albifrons – J. praehirsuta* mixed population with some intermediate male phenotypes in the Isles of Scilly, UK, an archipelago that is located more than 400 Km away across the English Channel (Fig. 2). This means that hybridization between these two species is probably much more widespread than previously thought (Solignac, 1969a, b, 1978). Perhaps more importantly, this also strongly suggests that hybridizing populations have been persisting for a long time.

In this study we identified 9 individuals (out of 110 males found in Normandy) showing intermediate morphological traits. While this figure depends on what one recognizes as morphologically pure or intermediate individuals, the majority of males clearly show strict *J. albifrons* or *J. praehirsuta* sexual traits despite the extensive genetic introgression demonstrated here (bimodal hybrid zone, Jiggins & Mallet, 2000). We concur with Solignac's observation (1978, p. 188) that the coexistence of the two morphs in spite of introgressive hybridization is of great interest, and we discuss below the mechanisms that may allow this coexistence in the long term and in repeated areas. This part of the discussion will focus on hybridizing populations only (that is, results from Normandy).

456 The closest population containing only *J. albifrons* that we found was located at more than 30
457 Km from the hybridizing populations. Moreover, the mixed populations that we found in this region
458 are geographically isolated from one another (there is most likely a discontinuity at least between
459 sites 7 and 8, Fig. 2). All species of the *Jaera albifrons* group have a direct development without a
460 dispersive larval phase, and in Normandy they do not live on seaweeds, which could potentially drift
461 across populations. Gravid females caught in the water column could occasionally be moved over a
462 great distance, but we did not find any *J. praehirsuta* outside of the mixed *J. albifrons* / *J. praehirsuta*
463 populations in Normandy. It is difficult to conduct an exhaustive survey over large areas for such
464 small species and we could have missed pure *J. albifrons* (and perhaps even *J. praehirsuta*)
465 populations at dispersal distance from our hybridizing populations, but we feel that it is unlikely.
466 Given that these crustaceans do not have a dispersive larval phase, and given the patchy distribution
467 of habitats, we infer from our surveys that hybridizing populations are replicated and patchily
468 distributed.
469 The hybridizing populations analysed in this study do not seem to be flanked by -or otherwise
470 functionally connected to- pure parental populations. They seem to be independent replicates of
471 hybridizing populations potentially distributed on a much larger geographical area than the one
472 studied here (e.g. on the UK coast). The influx of individuals from pure parental populations of *J.*
473 *praehirsuta* (and probably *J. albifrons*) is thus most likely not one of the forces acting to stabilize the
474 system. This interpretation needs further testing (e.g. from additional surveys and analyses of spatial
475 genetic structure), since if this hypothesis is confirmed, it would exclude dispersal-dependent models
476 of hybrid zones, chief among them the tension zone model (Barton & Hewitt, 1985), which relies on a
477 balance between immigration of parental genotypes and selection against hybrids. An alternative
478 hybrid zone model without immigration from parental populations involves ecological variation and
479 an advantage of hybrids in intermediate habitats (Moore, 1977). This hypothesis seem also be
480 excluded in our case because we were unable to detect any variation in habitat within hybridizing
481 populations (there was no identifiable variation in the distribution of *J. albifrons*-like, *J. praehirsuta*-

like, and morphologically intermediate individuals within a site). Other classical models are also inappropriate, for they combine the tension zone balance with ecological variation, either through environmentally-induced selection against hybrids (Endler, 1977) or a patchy distribution of habitats favouring one or the other species (mosaic hybrid zones, Harrison & Rand, 1989). The literature is also rich in empirical studies of hybridizing populations that do not fit one of these classical models, but it seems that situations where species coexist in spite of extensive introgression (i.e. bimodal hybrid zones) most often involve either an income of individuals from pure parental populations or ecological variation and habitat specialization within hybrid zones (or both). When incompletely isolated species occupy different ecological niches, comparative analyses of replicate hybrid zones have made quite clear that the maintenance of bimodality is correlated with the opportunity for ecological specialization (e.g. Culumber et al., 2011, Gagnaire et al., 2013). In the *Jaera albifrons* / *J. praehirsuta* system, the two species are more differentiated in our populations from Brittany where they specialize in two different habitats (rocks *vs* seaweeds) but, interestingly, parental forms coexist despite introgression in *hybridizing populations* in Normandy where there is probably no habitat specialization.

Assuming that there is no immigration from pure parental populations and no ecological variability, what evolutionary forces would allow pure *J. albifrons* and *J. praehirsuta* phenotypes to coexist in hybridizing populations? Past work suggests two strong candidates. First, the strongest isolating barrier between species of the *Jaera albifrons* complex is sexual isolation (Solignac, 1981). The courtship behavior of males (plus perhaps unknown male characteristics such as pheromone production or other unnoticed phenotypic variation) and female preference may still partially isolate *J. albifrons* from *J. praehirsuta* in hybridizing populations. One compelling hypothesis in this regard is that females of one species accept heterospecific mating more readily than females of the alternate species. This is nearly the rule in case of behavioral isolation (e.g. Coyne & Orr, 2004) and empirical tests suggest that this happens in the hybridizing populations studied here (although with a limited sample size, Bocquet & Solignac, 1969). The hypothesis that sexual isolation is a strong component is

508 also in line with other examples where hybrid zones remain bimodal (reviewed in Jiggins & Mallet,
509 2000).
510 The second candidate is selection against hybrids. This hypothesis is supported by Solignac
511 (1978, p. 186), who reported a strong hybrid breakdown in experimental F2 and backcrosses using
512 individuals from the hybridizing population of Luc-sur-Mer, Normandy. This suggests that the *J.*
513 *albifrons* / *J. praehirsuta* hybridizing populations documented in this study persist through a balance
514 between hybridization *versus* partial (perhaps asymmetrical) sexual isolation and selection against
515 certain recombined genotypes. This situation seems to be infrequent and the conditions of
516 persistence for such a system deserve further inquiry. In particular, it is a potential empirical example
517 of the model proposed by M'Gonigle *et al*. (2012), in which sexual selection, spatial variation in local
518 carrying capacity, and female mate-search costs allow partially divergent species to persist despite
519 ecological equivalence. More generally, it may provide some insight into the debated role of sexual
520 selection and sexual isolation in species divergence and coexistence. Yet it is remarkable that *J.*
521 *albifrons* and *J. praehirsuta* coexist in the long term despite their seemingly ecological equivalence. It
522 is possible that some unknown frequency-dependent mechanism (e.g. via an action of pathogens or
523 parasites) is acting to lower the likelihood of extinction of one or the two morphs.
524
525 5-Hybridizing and non-hybridizing mixed populations
526 Why do *J. praehirsuta* and *J. albifrons* hybridize when in contact in some populations and not
527 in others? There are two obvious differences between our studied populations from Brittany and
528 Normandy. First, where the two species were found to be non-hybridizing, they live on clearly
529 different habitats (under rocks vs on brown algae). The two habitats are located centimetres to
530 meters away, and a few individuals of each species were found on the habitat favoured by the other
531 species, meaning that *J. albifrons* and *J. praehirsuta* meet each other frequently in these sites (this is
532 why we call them mixed, or sympatric, populations: the two habitats are not separated by a large
533 geographical distance and can be reached by dispersing individuals of either species). Yet these two

habitats are radically different and are bound to impose a serious barrier to gene flow between species (ecological isolation). While neither species is restricted to one of these habitats in other parts of their range, the availability of distinct habitats may play an important role facilitating the coexistence of the two species and reducing hybridization opportunities. Appropriate seaweeds can also be found in hybridizing populations from Normandy, but they may not represent a suitable habitat there (e.g. because of wave exposure). Whatever the reason, there are no *Jaera* on algae in the hybridizing populations reported here. One plausible hypothesis is thus that ecological diversification facilitates the coexistence and divergence of *J. albifrons* and *J. praehirsuta* wherever this is possible. Another interesting idea, suggested by an anonymous reviewer, is that hybridization allows the hybrid and subsequently *J. praehirsuta* to acquire adaptations to the array of challenges presented in switching from a seaweed to a rocky habitat. One avenue of research to tackle these questions will be to search for pure *J. praehirsuta* populations occupying rocky habitats (and *J. albifrons* populations on seaweeds).

The second difference between hybridizing and reproductively isolated populations is their geographic location. Contrary to the idea that natural hybridization is the exception and not the rule in this system (Solignac, 1969a, b, 1978), we suggest that hybridizing populations may be found in a large geographic area. It is therefore possible that populations from two large geographic zones (one encompassing Normandy and the other one encompassing Brittany) have a distinct demographic and evolutionary history (e.g. with variations in the conditions of secondary contacts between species after periods of isolation). The intra-specific genetic differentiation between regions appeared to be similar in *J. albifrons* and *J. praehirsuta* ($F_{ST}$ = 0.14 and 0.15, respectively) and similar in intensity to the genetic differentiation between species observed in Brittany. This is also apparent on the PCA where the first axis partitions the genotypes in function of their geographic origin (Brittany on the left, Normandy on the right, Fig. 5).

Testing these ideas will require analyzing the genetic structure of the two species over a large geographical scale and surveying mixed populations for morphological or genetic signs of hybridization in different habitat conditions.

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**Data accessibility**

Multi-locus genotypes at each sampling location will be made available in Dryad.

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697

**Table 1:** *Jaera albifrons and J. praehirsuta* sampling locations, sample sizes, and genetic diversity (observed and expected heterozygosity $H_o$ and $H_e$, and $F_{IS}$). Individuals with an intermediate phenotype ($n$=9) from sites 7 and 8 are not included. The statistical significance of $F_{IS}$ is indicated (*: $p$-value < 0.05)

| Sampled site | | Coordinates | *J. albifrons* | | | | *J. praehirsuta* | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *N* | $H_o$ | $H_e$ | $F_{IS}$ | *N* | $H_o$ | $H_e$ | $F_{IS}$ |
| BRITTANY | | | | | | | | | | |
| | 1 | 48°40'27.6"N, 3°57'11.3"W | 24 | 0.48 | 0.55 | 0.137* | 10 | 0.53 | 0.61 | 0.145 |
| | 2 | 48°40'20.9"N, 3°57'00.3"W | 25 | 0.47 | 0.57 | 0.188* | 7 | 0.55 | 0.64 | 0.142 |
| | 3 | 48°39'10.8"N, 3°57'03.0"W | 22 | 0.51 | 0.54 | 0.070 | 18 | 0.54 | 0.62 | 0.135* |
| | 4 | 48°39'12.3"N, 3°57'00.4"W | 24 | 0.5 | 0.57 | 0.131* | 14 | 0.52 | 0.62 | 0.168* |
| | 5 | 48°39'34.1"N, 3°56'25.7"W | 25 | 0.48 | 0.55 | 0.115 | 5 | 0.63 | 0.69 | 0.097 |
| | 6 | 48°39'33.5"N, 3°56'31.2"W | 22 | 0.45 | 0.52 | 0.132* | 26 | 0.5 | 0.58 | 0.153* |
| NORMANDY | | | | | | | | | | |
| | 7 | 49°23'30.4"N, 1°02'09.6"W | 20 | 0.49 | 0.52 | 0.059 | 12 | 0.45 | 0.53 | 0.163* |
| | 8 | 49°21'15.7"N, 0°47'54.2"W | 9 | 0.42 | 0.48 | 0.141 | 44 | 0.52 | 0.58 | 0.107* |
| | 9 | 49°20'53.5"N, 0°41'03.2"W | 15 | 0.43 | 0.47 | 0.094 | 1 | - | - | - |

**Table 2:** Distribution of the genetic variation estimated through hierarchical analyses of molecular variance in regions Brittany and Normandy (as defined in Fig. 1). We present the results obtained with and without loci Ja41 and Ja64, which show a very strong differentiation in Normandy (see $F_{CT}$ at these two loci).

| Source of variation | **Brittany** | | | **Normandy** | | |
|---|---|---|---|---|---|---|
| | % of total variation | F-stat | p-value | % of total variation | F-stat | p-value[a] |
| **23 loci** | | | | | | |
| Among sampling sites | | | | | | |
| between species | 13.2 | $F_{CT}$ = 0.132 | $p$ = 0.001 | 7.4 | $F_{CT}$ = 0.074 | ($p$ = 0.336) |
| within species | 1.3 | $F_{SC}$ = 0.015 | $p$ < 0.001 | 1.5 | $F_{SC}$ = 0.016 | $p$ = 0.003 |
| Within sampling sites | | | | | | |
| among individuals | 9.7 | $F_{IS}$ = 0.114 | $p$ < 0.001 | 8.8 | $F_{IS}$ = 0.097 | $p$ < 0.001 |
| within individuals | 75.8 | | $p$ < 0.001 | 82.3 | | $p$ < 0.001 |
| **21 loci (without Ja41 & Ja64)** | | | | | | |
| Among sampling sites | | | | | | |
| between species | 12.5 | $F_{CT}$ = 0.125 | $p$ = 0.002 | 1.7 | $F_{CT}$ = 0.017 | ($p$ = 0.328) |
| within species | 1.2 | $F_{SC}$ = 0.013 | $p$ < 0.001 | 1.8 | $F_{SC}$ = 0.019 | $p$ = 0.001 |
| Within sampling sites | | | | | | |
| among individuals | 10.1 | $F_{IS}$ = 0.117 | $p$ < 0.001 | 8.4 | $F_{IS}$ = 0.087 | $p$ < 0.001 |
| within individuals | 76.3 | | $p$ < 0.001 | 88.1 | | $p$ < 0.001 |
| **2 loci (Ja41 & Ja64)** | | | | | | |
| Among sampling sites | | | | | | |
| between species | 20.2 | $F_{CT}$ = 0.202 | $p$ = 0.003 | 42.8 | $F_{CT}$ = 0.428 | ($p$ = 0.332) |
| within species | 2.5 | $F_{SC}$ = 0.032 | $p$ < 0.001 | -0.7 | $F_{SC}$ = -0.012 | $p$ = 0.65 |
| Within sampling sites | | | | | | |
| among individuals | 8.9 | $F_{IS}$ = 0.115 | $p$ < 0.001 | 11.6 | $F_{IS}$ = 0.2 | $p$ < 0.001 |
| within individuals | 68.4 | | $p$ < 0.001 | 46.3 | | $p$ < 0.001 |

a) The statistical significance of between-species variation was tested using permutations of sites between species, which is essentially powerless in region Normandy where only two sites harboring mixed populations were found. The relevant p-values (indicated in grey) are thus meaningless, and the differentiation between species was better tested in this case using non-hierarchical F-statistics within each site (see text).

**Table 3:** Pairwise genetic differentiation between populations in Brittany. Above diagonal: pairwise $F_{ST}$. Below diagonal: exact test of population differentiation *p*-value (in bold when significant). Values in the grey area correspond to inter-specific $F_{ST}$.

| | | *J. albifrons* | | | | | | *J. praehirsuta* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 |
| *J. albifrons* | | | | | | | | | | | | | |
| | 1 | - | 0.02 | 0.061 | 0.06 | 0.035 | 0.034 | 0.178 | 0.161 | 0.14 | 0.14 | 0.062 | 0.167 |
| | 2 | **0.003** | - | 0.066 | 0.055 | 0.036 | 0.05 | 0.175 | 0.151 | 0.137 | 0.141 | 0.059 | 0.161 |
| | 3 | **0** | **0** | - | 0.005 | 0.021 | 0.017 | 0.182 | 0.177 | 0.155 | 0.149 | 0.099 | 0.187 |
| | 4 | **0** | **0** | 0.190 | - | 0.015 | 0.024 | 0.174 | 0.167 | 0.141 | 0.14 | 0.092 | 0.179 |
| | 5 | **0** | **0** | **0.003** | **0.014** | - | 0.009 | 0.207 | 0.195 | 0.17 | 0.166 | 0.1 | 0.196 |
| | 6 | **0** | **0** | **0.008** | **0.001** | 0.116 | - | 0.201 | 0.184 | 0.157 | 0.152 | 0.103 | 0.19 |
| *J. praehirsuta* | | | | | | | | | | | | | |
| | 1 | **0** | **0** | **0** | **0** | **0** | **0** | - | -0.007 | 0.01 | -0.005 | -0.048 | 0.017 |
| | 2 | **0** | **0** | **0** | **0** | **0** | **0** | 0.671 | - | -0.005 | -0. 004 | -0.068 | 0.008 |
| | 3 | **0** | **0** | **0** | **0** | **0** | **0** | 0.160 | 0.715 | - | -0.008 | -0.061 | 0.004 |
| | 4 | **0** | **0** | **0** | **0** | **0** | **0** | 0.737 | 0.657 | 0.919 | - | -0.074 | 0.005 |
| | 5 | **0.004** | **0.003** | **0** | **0** | **0** | **0** | 0.979 | 0.988 | 0.999 | 0.999 | - | -0.075 |
| | 6 | **0** | **0** | **0** | **0** | **0** | **0** | **0.042** | 0.278 | 0.295 | 0.268 | 1 | - |

**Table 4**: Pairwise genetic differentiation between populations in Normandy. Above diagonal: pairwise $F_{ST}$. Below diagonal: exact test of population differentiation *p*-value (in bold when significant). Values in the grey area correspond to inter-specific $F_{ST}$.

| | *J. albifrons* | | *J. praehirsuta* | |
|---|---|---|---|---|
| | 7 | 8 | 7 | 8 |
| *J. albifrons* | | | | |
| 7 | - | 0.025 | 0.104 | 0.073 |
| 8 | **0.037** | - | 0.153 | 0.101 |
| *J. praehirsuta* | | | | |
| 7 | **0** | **0** | - | 0.018 |
| 8 | **0** | **0** | **0.015** | - |

**Figure legends**

**Figure 1**: Morphological differentiation at peraeopods (numbered P1-P7) between males *Jaera albifrons* (carpus of peraeopods P6 and P7 extended as a lobe with a number of straight setae) and *J. praehirsuta* (peraeopods P1-4 with many curved setae) as found in region Brittany (western France, drawings modified from Solignac, 1981). A few individuals with secondary sexual traits intermediate to *J. albifrons* and *J. praehirsuta* were found in region Normandy. The length of the individual (a female) represented on the picture is 4 mm. Photography credit to Guillaume Evanno & Thomas Broquet.

**Figure 2:** Sampling sites in two regions of Western France ("Brittany" and "Normandy"). Pie charts give the number of males showing secondary sexual traits typical of *Jaera albifrons* (in blue), *Jaera praehirsuta* (in green), and intermediate phenotypes (in red) sampled at each location. Note that these numbers reflect the relative proportions of each species at each site in Normandy, where the two species occupy the same microhabitat and cannot be distinguished in the field. By contrast, in Brittany pie charts are representative of sample sizes but not necessary of the relative density of each species (because there the two species occupy two different habitats with little overlap, see text). Mixed populations from Normandy were found following an intensive survey (geographic extent shown in yellow, details in text), revealing that the hybridizing population originally studied by Michel Solignac in 1965-1970 (Luc-sur-Mer, indicated by a star) is now extinct. The nearest non-mixed populations that we could find comprised only *Jaera albifrons* (empty circles). Additional mixed populations with some individuals showing an intermediate phenotype were further found in Yport and the Isles of Scilly (black dots, see text).

**Figure 3**: Locus-by-locus genetic differentiation between *J. albifrons* and *J. praehirsuta* ($F_{CT}$, expressed in this figure as the percentage of genetic variation due to differences between species) in Brittany (black dots) and in Normandy (white dots). Significant differentiation between $F_{CT\text{-}Brittany}$ and $F_{CT\text{-}Normandy}$ is represented by a star above the locus name. Here the loci are arranged by increasing order of $F_{CT\text{-}Normandy}$. We see that all white dots but two lie near zero (no differentiation between species in Normandy) while the two remaining loci (Ja41 and Ja64) show a strong differentiation. These two microsatellites are the only loci showing a significantly stronger differentiation between species in Normandy than in Brittany (white vs black dots). In Brittany the average level of differentiation between species is near 13% and there is also heterogeneity across loci.

**Figure 4**: STRUCTURE clusters (K=2) defined in Brittany (A) and Normandy (B). Because loci Ja41 and Ja64 are outliers in Normandy (see text and Fig. 3), STRUCTURE results for this region are presented with all loci (23 loci, panel B1), without the two outliers (21 loci, panel B2), and considering only Ja41 and Ja64 (2 loci, panel B3). Numbers refer to sampling sites (Figure 2). We see that individuals morphologically identified as *J. albifrons* or *J. praehirsuta* cluster into two distinct groups in Brittany (regardless of sampling location, panel A), while this will remain true in Normandy only due to the effect of two markers out of twenty-three (panels B1, B2 and B3).

**Figure 5**: Principal component analysis based on individual multi-locus genotypes at 23 microsatellite loci. The first axis separates individuals from Brittany (left) and Normandy (right). The second axis shows the genetic divergence between males bearing sexual traits typical of *J. albifrons* vs *J. praehirsuta*. We see that these two types of males are less genetically differentiated in region Normandy (in agreement with results from the analysis of molecular variance and STRUCTURE results from figure 4) and males with an intermediate phenotype are undifferentiated from *J. praehirsuta*.

Figure 1

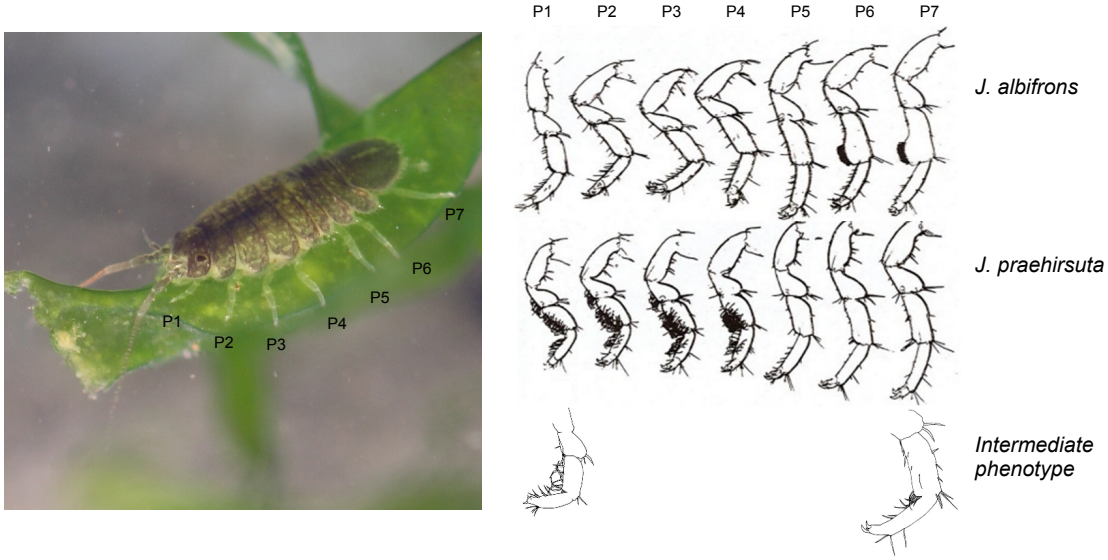

P1
P2
P3
P4
P5
P6
P7
P7
P6
P5
P4
P1
P2
P3
J. albifrons
J. praehirsuta
Intermediate phenotype

Figure 2

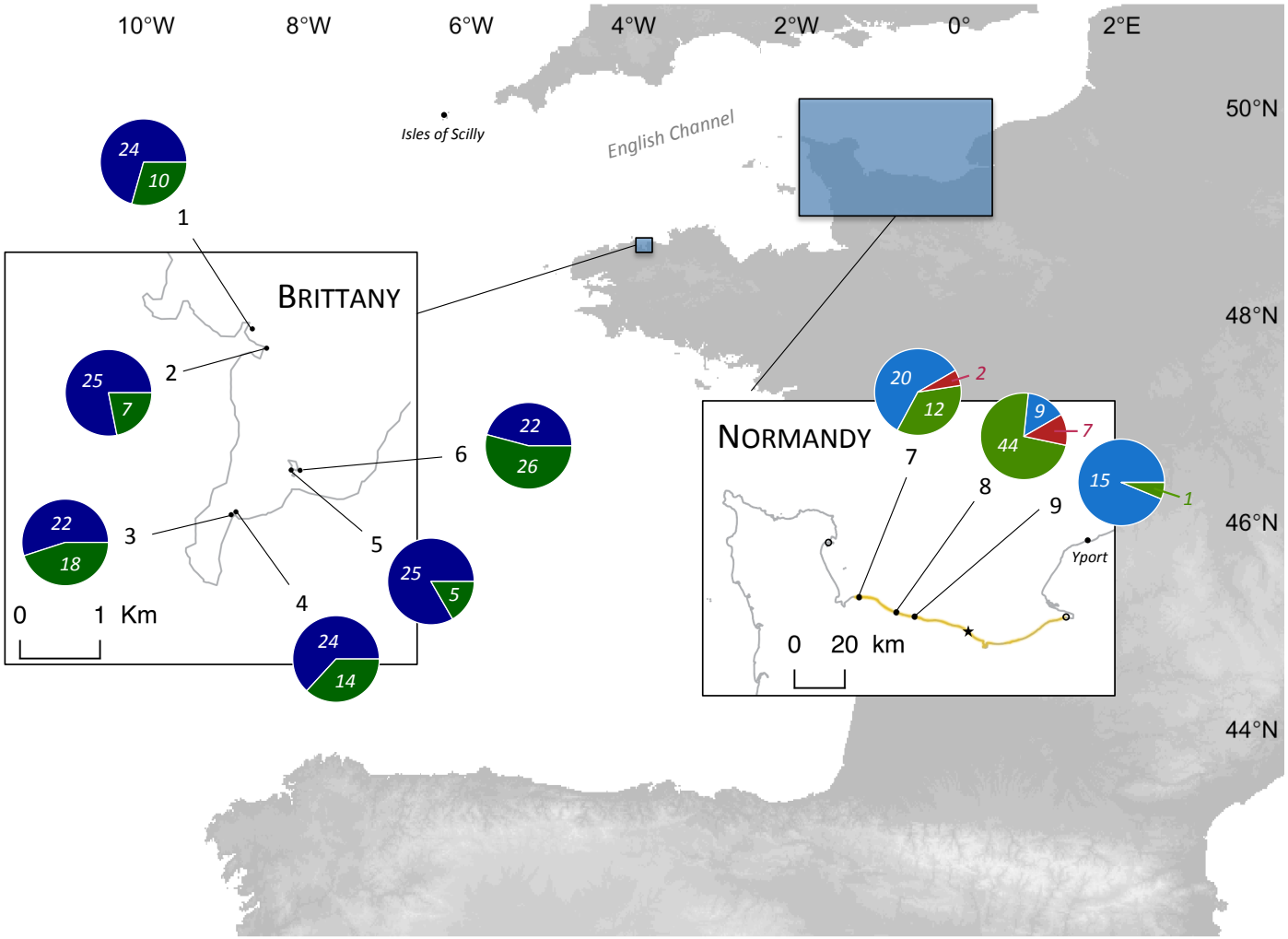

10°W
8°W
6°W
4°W
2°W
0°
2°E
50°N
48°N
46°N
44°N
Isles of Scilly
English Channel
BRITTANY
NORMANDY
Yport
0 1 Km
0 20 km
1
2
3
4
5
6
7
8
9
24
10
25
7
22
18
22
26
25
5
24
14
20
12
2
9
44
7
15
1

Figure 3

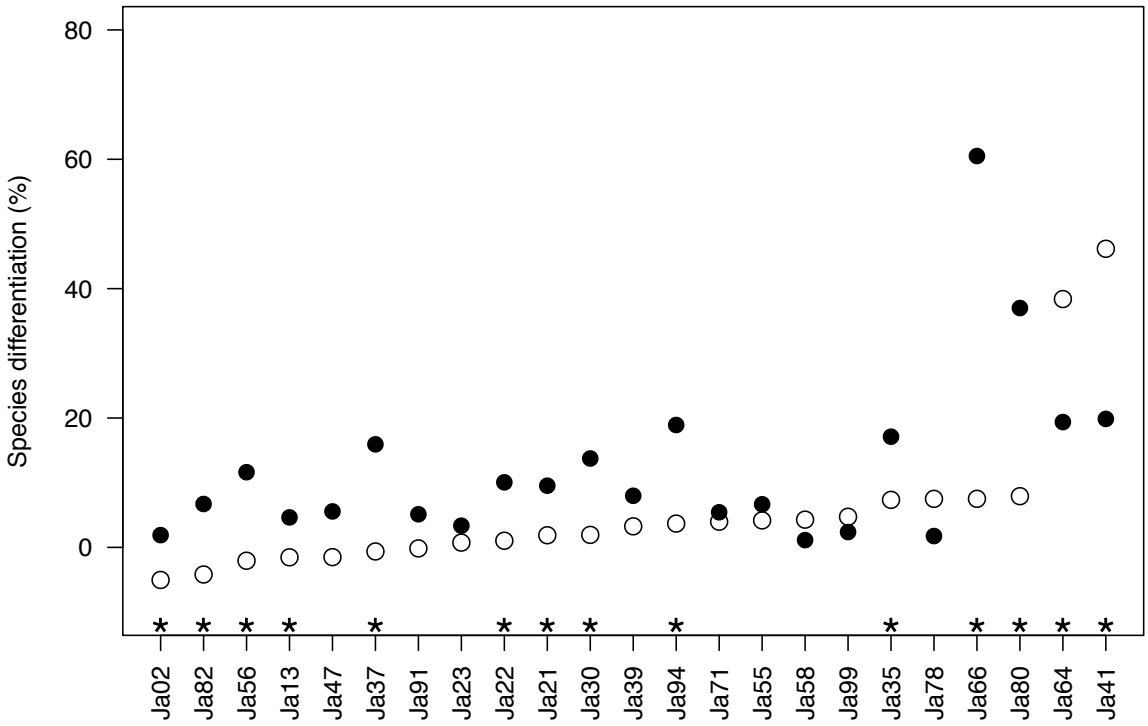

Species differentiation (%)
80
60
40
20
0
Ja02
Ja82
Ja56
Ja13
Ja47
Ja37
Ja91
Ja23
Ja22
Ja21
Ja30
Ja39
Ja94
Ja71
Ja55
Ja58
Ja99
Ja35
Ja78
Ja66
Ja80
Ja64
Ja41

Figure 4

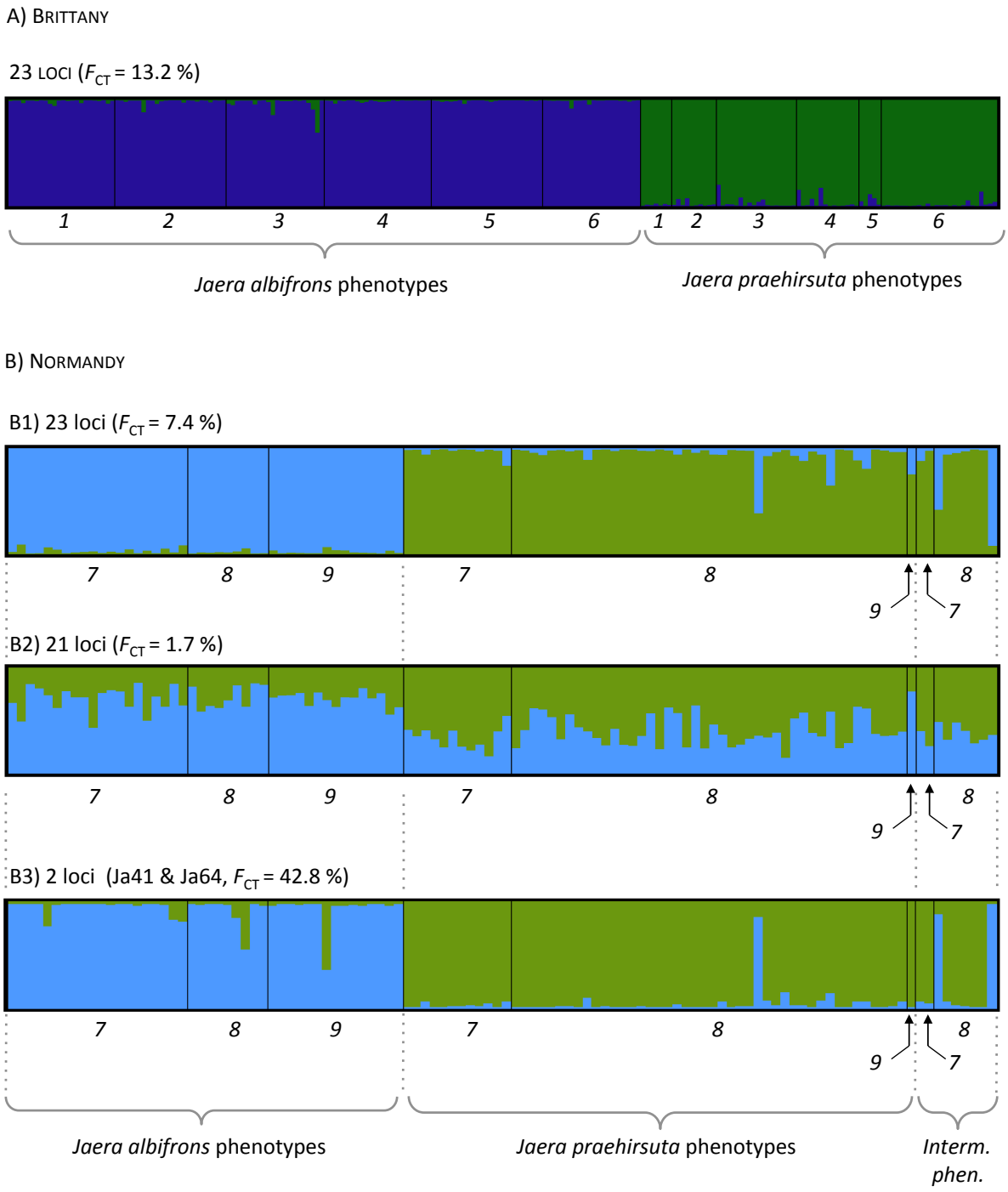
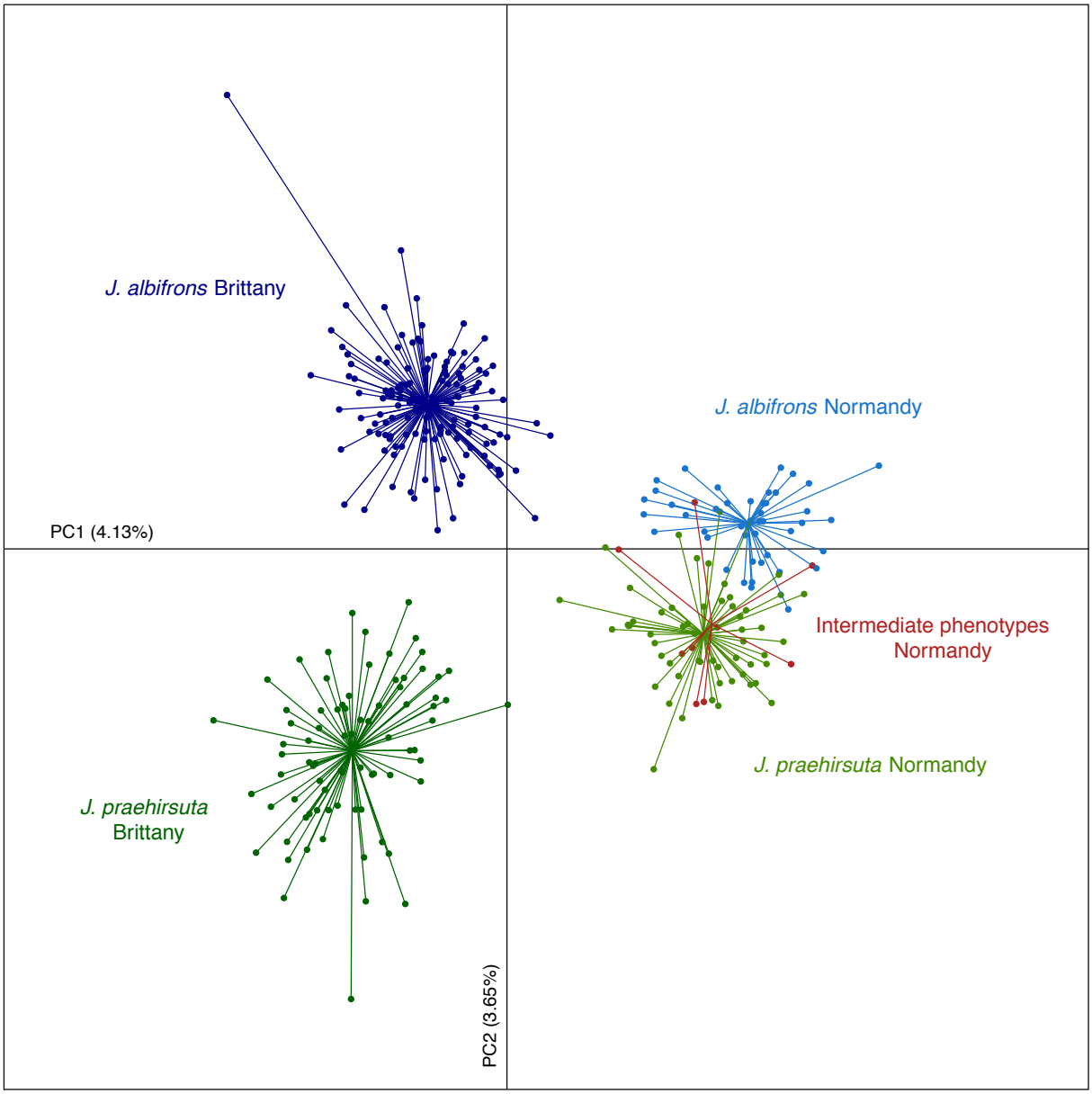

A) BRITTANY
23 LOCI ($F_{CT}$ = 13.2 %)
1 2 3 4 5 6 1 2 3 4 5 6
Jaera albifrons phenotypes
Jaera praehirsuta phenotypes
B) NORMANDY
B1) 23 loci ($F_{CT}$ = 7.4 %)
7 8 9 7 8 9 7 8
B2) 21 loci ($F_{CT}$ = 1.7 %)
7 8 9 7 8 9 7 8
B3) 2 loci (Ja41 & Ja64, $F_{CT}$ = 42.8 %)
7 8 9 7 8 9 7 8
Jaera albifrons phenotypes
Jaera praehirsuta phenotypes
Interm. phen.

Figure 5


J. albifrons Brittany
J. albifrons Normandy
Intermediate phenotypes
Normandy
J. praehirsuta Normandy
J. praehirsuta
Brittany
PC1 (4.13%)
PC2 (3.65%)

**Supplementary material**

**Figure S1**: Distribution of individuals along the coast at Ste-Honorine-des-Pertes (site 8, Normandy) during a survey realized in summer 2014. Using a precise localization system (5.5 cm horizontal accuracy), we found that male individuals bearing sexual traits typical of *J. albifrons* or *J. praehirsuta* occupied the same microhabitats and show no particular distribution patterns (both species are intermixed with no apparent distributional gradient). Females were included in this analysis, and some of them were identified as *J. albifrons* or *J. praehirsuta* based on the sexual characters born by their sons (raised in the lab). For clarity purpose we did not distinguish males and females in this figure. Note also that many individuals are not visible here due to the near superposition of their locations.

**Figure S2:** Bootstrap distribution of $F_{CT}$ (genetic differentiation between species) in Brittany (black) and Normandy (white) at loci Ja41 (Panel A) and Ja64 (B). Here we see that 1000 resampling iterations rarely produced a situation where $F_{CT\text{-}Normandy}$ was greater or equal to $F_{CT\text{-}Brittany}$ at these two loci ($p$-value ≤ 0.001). Observed $F_{CT}$ are indicated by triangles (Brittany: black, Normandy: white).

**Figure S3:** Bootstrap distribution of $F_{CT}$ (genetic differentiation between species) in Brittany (black) and Normandy (white) at 23 microsatellite loci. Observed $F_{CT}$ in each region are indicated by triangles.

**Figure S4:** Allelic frequencies at locus Ja41 in *Jaera albifrons* (blue) and *Jaera praehirsuta* (green) from Brittany (panel A) and Normandy (panel B). Note that the $F_{CT}$ values reported here were calculated in hierarchical analyses of variance without the individuals showing an intermediate phenotype (see main text).

**Figure S5:** Allelic frequencies at locus Ja64 in *Jaera albifrons* (blue) and *Jaera praehirsuta* (green) from Brittany (panel A) and Normandy (panel B). Note that the $F_{CT}$ values reported here were calculated in

hierarchical analyses of variance without the individuals showing an intermediate phenotype (see main text).

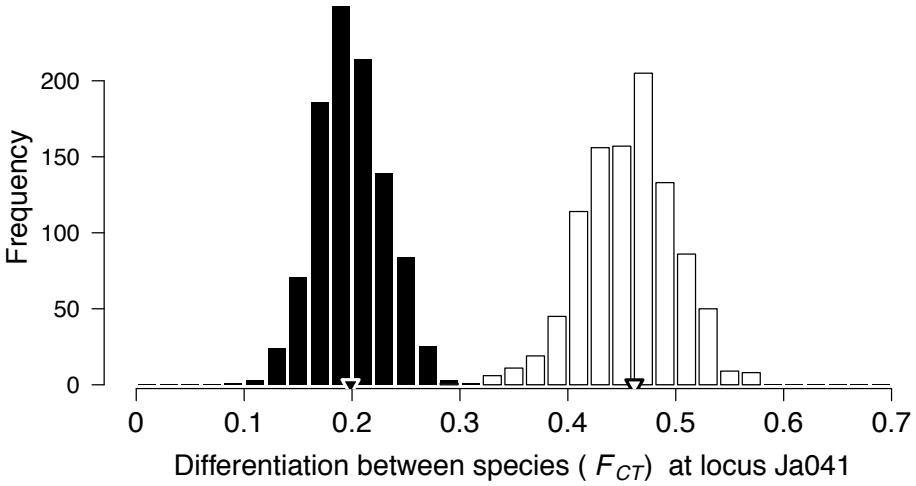
**Figure S6**: Patterns of isolation-by distance observed in Brittany for *Jaera albifrons* (blue circles; y=0.0209x +0.0011, $R^2 = 0.73$, Mantel test *p*-value < 0.01) and *J. praehirsuta* (green circles; y=0.0065x -0.0133, $R^2=0.11$, Mantel test *p*-value = 0.02).

Figure S1


Jaera albifrons
Jaera praehirsuta
Intermediate phenotype
Unidentified female
N
0
50
100 m

Figure S2

A


Frequency
200
150
100
50
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Differentiation between species ( $F_{CT}$) at locus Ja041

B

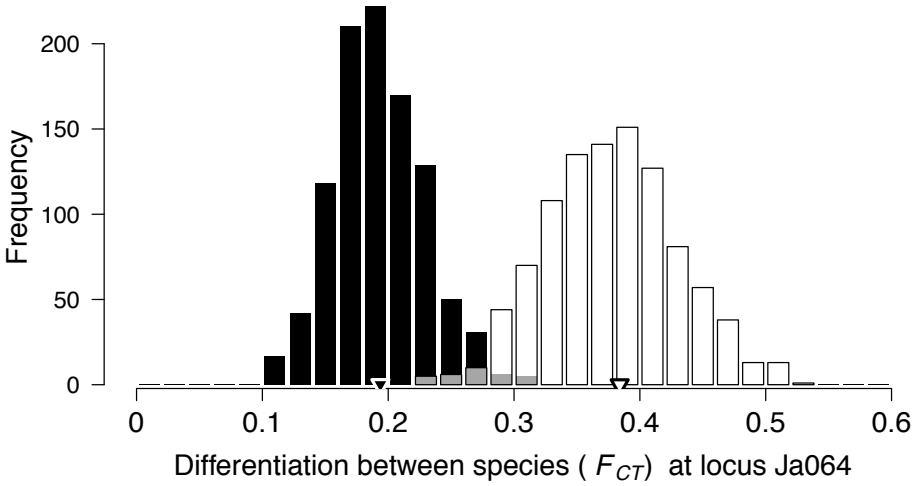
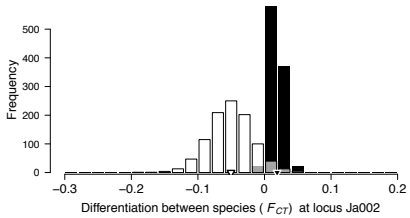
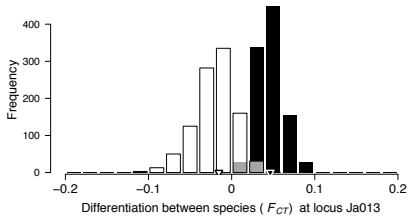
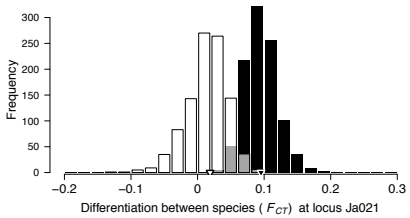
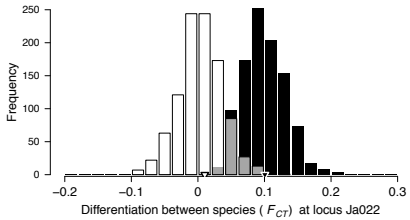

Frequency
200
150
100
50
0
0
0.1
0.2
0.3
0.4
0.5
0.6
Differentiation between species ( $F_{CT}$) at locus Ja064

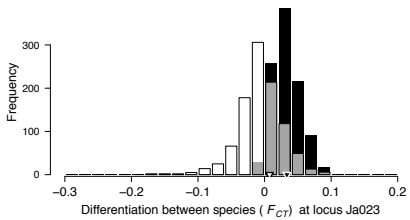
Figure S3

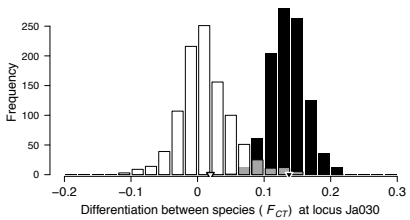

Frequency
Differentiation between species ( $F_{CT}$) at locus Ja002

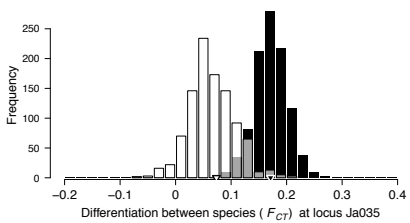

Frequency
Differentiation between species ( $F_{CT}$) at locus Ja013

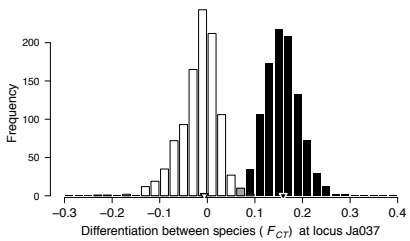

Frequency
Differentiation between species ( $F_{CT}$) at locus Ja021

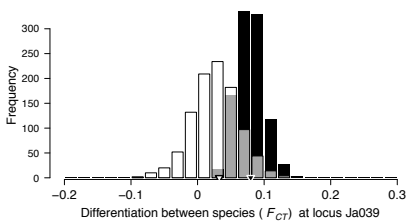

Frequency
Differentiation between species ( $F_{CT}$) at locus Ja022

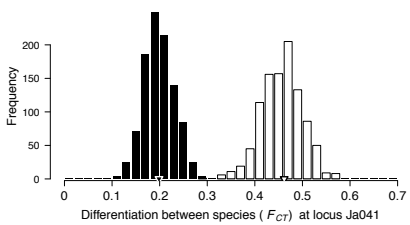

Frequency
Differentiation between species ( $F_{CT}$) at locus Ja023

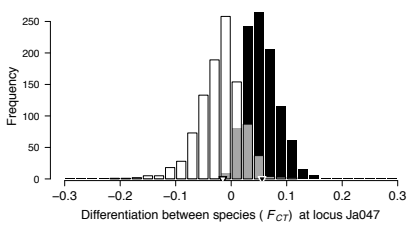

Frequency
Differentiation between species ( $F_{CT}$) at locus Ja030


Frequency
Differentiation between species ( $F_{CT}$) at locus Ja035


Frequency
Differentiation between species ( $F_{CT}$) at locus Ja037


Frequency
Differentiation between species ( $F_{CT}$) at locus Ja039


Frequency
Differentiation between species ( $F_{CT}$) at locus Ja041


Frequency
Differentiation between species ( $F_{CT}$) at locus Ja047

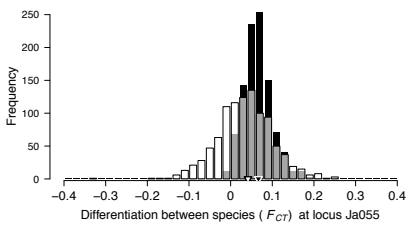
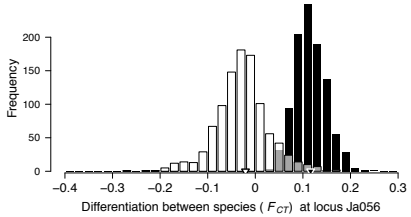
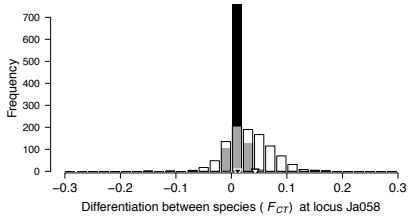
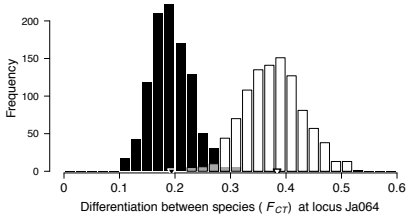
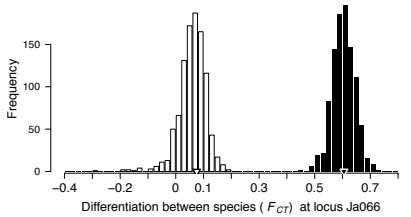

Frequency
Differentiation between species ( $F_{CT}$) at locus Ja055

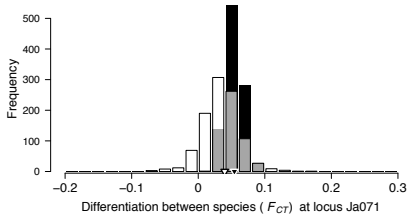
Figure S3 (continued)

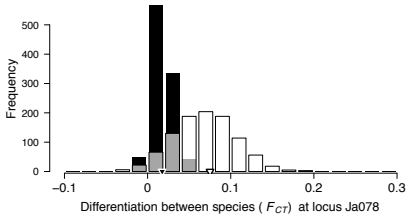


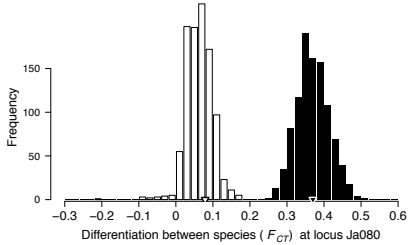



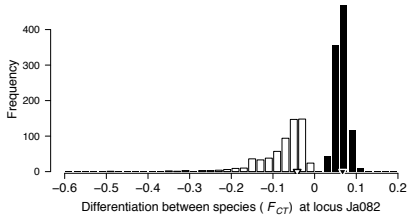



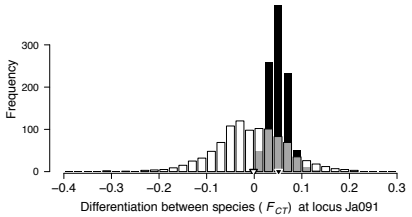



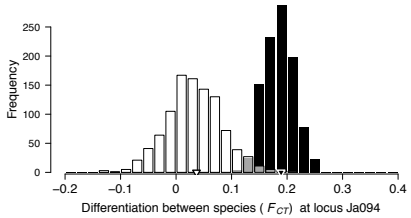



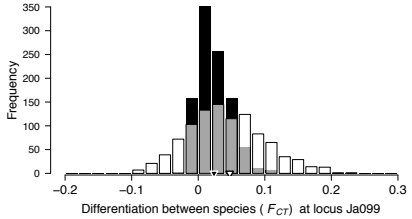



Figure S4

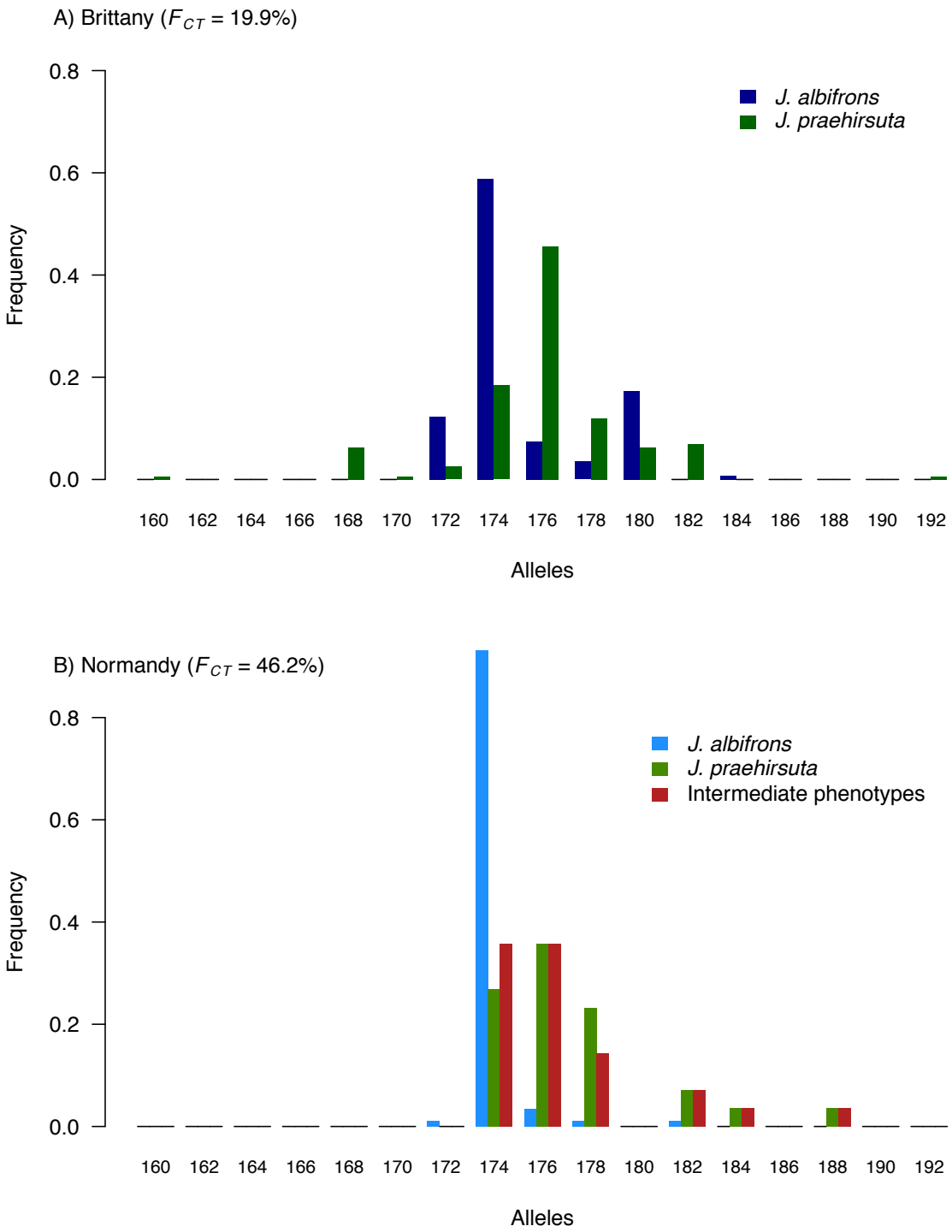

A) Brittany ($F_{CT}$ = 19.9%)
0.8
0.6
0.4
0.2
0.0
Frequency
J. albifrons
J. praehirsuta
160 162 164 166 168 170 172 174 176 178 180 182 184 186 188 190 192
Alleles
B) Normandy ($F_{CT}$ = 46.2%)
0.8
0.6
0.4
0.2
0.0
Frequency
J. albifrons
J. praehirsuta
Intermediate phenotypes
160 162 164 166 168 170 172 174 176 178 180 182 184 186 188 190 192
Alleles

Figure S5

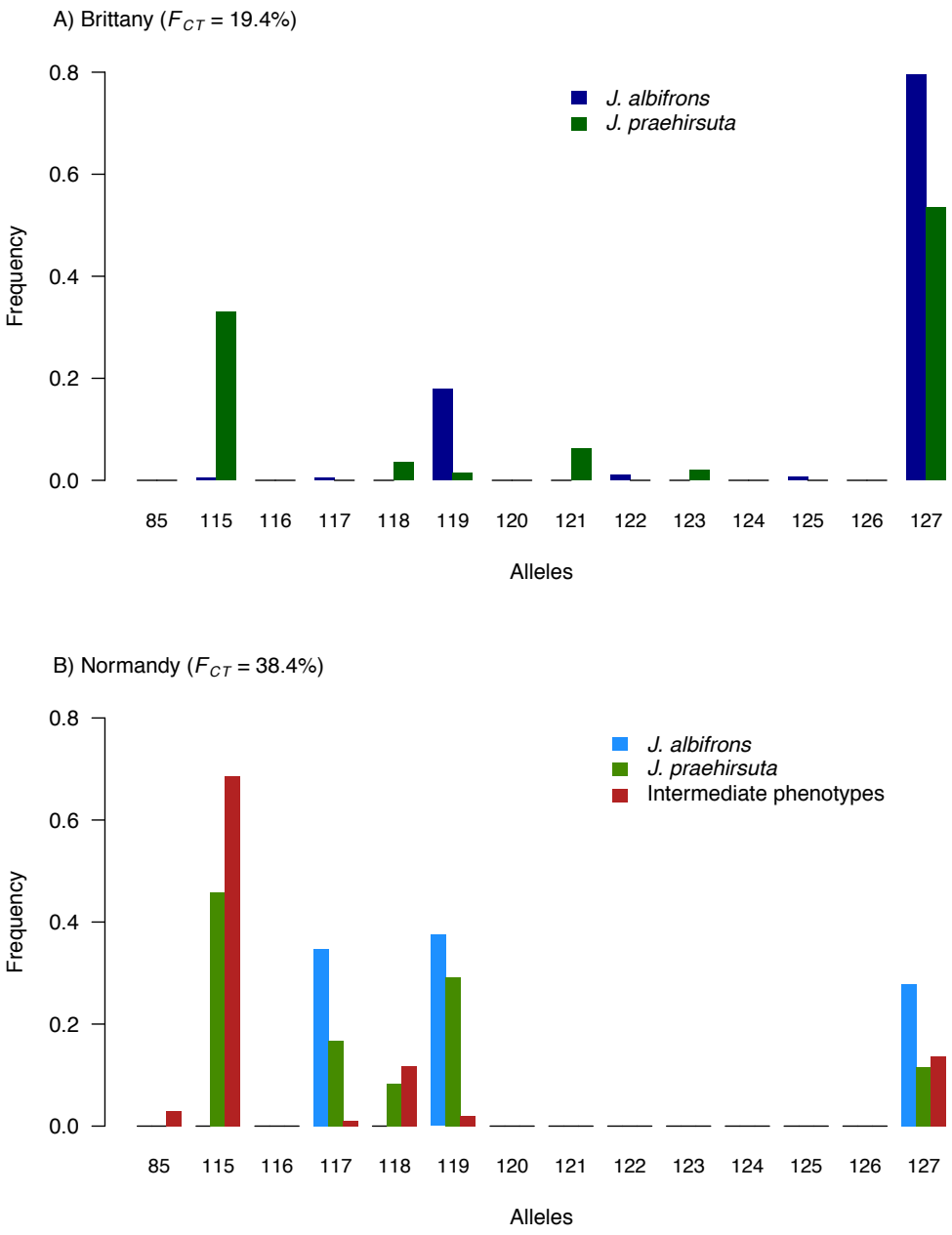
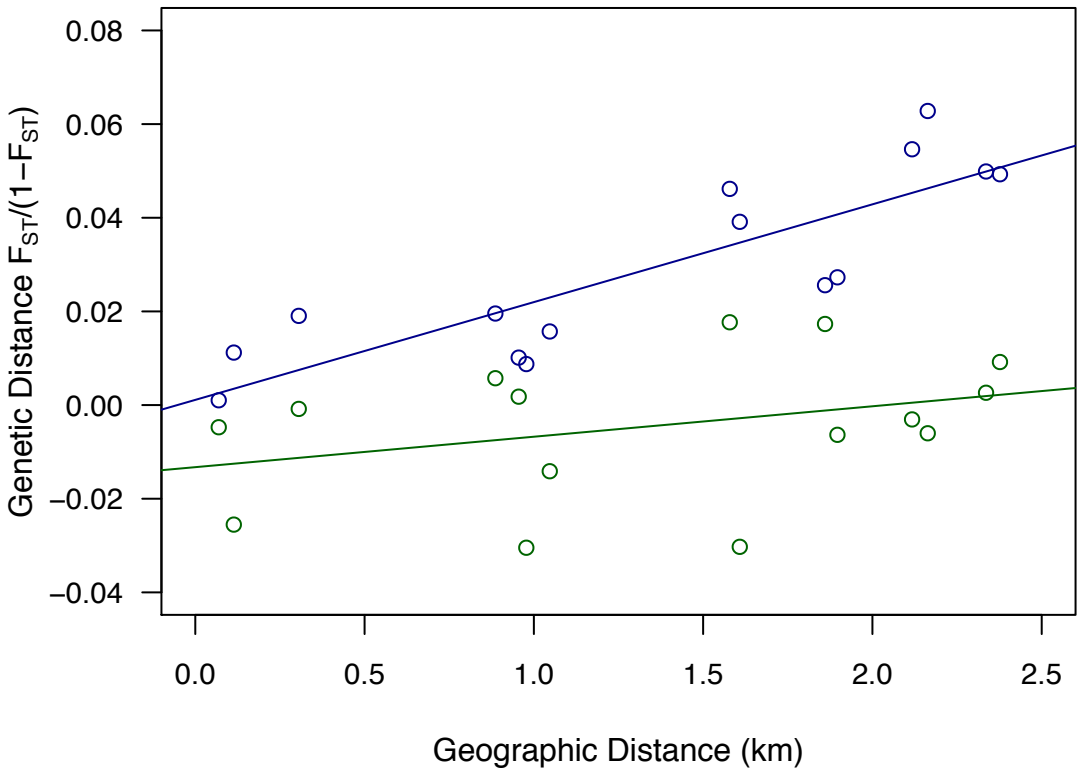

A) Brittany ($F_{CT}$ = 19.4%)
0.8
0.6
0.4
0.2
0.0
Frequency
J. albifrons
J. praehirsuta
85
115
116
117
118
119
120
121
122
123
124
125
126
127
Alleles
B) Normandy ($F_{CT}$ = 38.4%)
0.8
0.6
0.4
0.2
0.0
Frequency
J. albifrons
J. praehirsuta
Intermediate phenotypes
85
115
116
117
118
119
120
121
122
123
124
125
126
127
Alleles

Figure S6


0.08
0.06
0.04
0.02
0.00
−0.02
−0.04
Genetic Distance $F_{ST}/(1-F_{ST})$
0.0
0.5
1.0
1.5
2.0
2.5
Geographic Distance (km)