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Genetic entities and mating system in hermaphroditic *Fucus spiralis* and its close dioecious relative *F. vesiculosus* (Fucaceae, Phaeophyceae)

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Abstract

To date, molecular markers have not settled the question of the specific status of the closely related, but phylogenetically unresolved, brown seaweeds, hermaphroditic *Fucus spiralis* and dioecious *Fucus vesiculosus*, nor their propensity for natural hybridization. To test the degree of species integrity and to assess effect of the mating system on the population genetic structure, 288 individuals coming from parapatric (discontinuous) and sympatric (contiguous) spatial configurations at two sites were genotyped with five microsatellite loci. Using a Bayesian admixture analysis, our results show that *F. spiralis* and *F. vesiculosus* comprise clearly distinct genetic entities (clusters) generally characterized by cosexual and unisexual individuals, respectively. Genetic diversity within each entity suggests that *F. spiralis* reproduces primarily through selfing while *F. vesiculosus* is characterized by an endogamous breeding regime. Nevertheless, aberrant sexual phenotypes were observed in each cluster, no diagnostic alleles were revealed and 10% of study individuals were intermediate between the two genetic entities. This pattern can be explained by recent divergence of two taxa with retention of ancestral polymorphism or asymmetrical, introgressive hybridization. However, given (i) coincident monomorphism at three loci in *spiralis* clusters and (ii) that significantly more intermediates were observed in sympatric stations than in parapatric stations, we argue that interspecific gene flow has occurred after divergence of the two taxa. Finally, we show that whether recently separated or recently introgressive, the divergent breeding systems probably contribute to species integrity in these two taxa.

Keywords: admixture and assignment analysis, Fucaceae, hybridization, marine alga, mating system, microsatellites

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Introduction

Changes in mating system directly influence the amount and distribution of genetic variation and can lead to reproductive isolation and eventually to speciation (Charlesworth & Charlesworth 1995; Hamrick & Godt 1997). Variability in the mating system can thus affect the movement of genes within and among conspecific populations (e.g. Costich & Meagher 1992; Dorken *et al*. 2002) and among closely related hybridizing species (Sweigart & Willis 2003). It is rare for both hermaphroditic and dioecious populations to be maintained within a species (Charlesworth 1999; for examples among land plants, see Dorken *et al*. 2002 and references therein), but many genera, in seaweeds and land plants alike, contain potentially hybridizing hermaphroditic and dioecious species (e.g. seaweeds, Bold & Wynne 1985; plants, Renner & Ricklefs 1995). For example, *Fucus spiralis* L. and *Fucus vesiculosus* L., two closely related, highly successful and ecologically important brown seaweed species share a wide distribution over northern Atlantic, Channel and North Sea shores. Typically, in marine environments, the two species grow in different, but proximate habitats: *F. spiralis* and *F. vesiculosus* are found in the upper intertidal and mid-intertidal zones, respectively.

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These two taxa show contrasting, species-diagnostic mating systems — *F. spiralis* individuals are hermaphroditic[1] whereas *F. vesiculosus* individuals are unisexual (i.e. dioecious taxon). Apart from sexual phenotype, gross morphological differences separate typical individuals of the two species, but extensive vegetative morphological continuity between *F. spiralis* and *F. vesiculosus* is documented (e.g. Burrows & Lodge 1951; Pérez-Ruzafa *et al*. 1993; Scott & Hardy 1994). Nevertheless, without consideration of sexual phenotype, the observation of morphologically intermediate plants in the field has been taken as evidence for natural hybridization between the two taxa (Stomps 1911; Kniep 1925; Burrows & Lodge 1951; Scott & Hardy 1994). However, there are few published accounts of successful controlled crosses between *F. spiralis* and *F. vesiculosus* (Kniep 1925); moreover, without genetic markers to confirm the hybrid nature of the progeny produced, contamination by intraspecific progeny cannot be ruled out. The taxonomic identity of and the potential for hybridization between *F. spiralis* and *F. vesiculosus* raise questions as to the stability, fate and role of contrasting mating systems in maintaining species integrity.

Fertilization is external in *Fucus* — both eggs and sperm are released into the environment before syngamy. Therefore, the potential for self-fertilization, and thus the advantages of hermaphroditism (Fisher 1941; Stebbins 1950; Baker 1955) are theoretically limited. Nonetheless, hermaphroditic individuals appear to be self-compatible as self-fertilization occurs *in vitro* in *F. spiralis* (Vernet & Harper 1980; Müller & Gassman 1984). Furthermore, due to the environmentally cued synchronization of gamete release with calm periods (Serrão *et al*. 1996; Berndt *et al*. 2002), the negative buoyancy of eggs and negative phototaxis of sperm (Brawley *et al*. 1999) and pheromonal gamete attractants (Müller & Gassman 1984), gamete dispersal is hypothesized to be spatially restricted. Field estimates in fucoid algae recorded that most eggs settle within *c.* 0.5 m of the source, although dispersal beyond 2–6 m may also occur (Serrão *et al*. 1997; Dudgeon *et al*. 2001). This low dispersal potential may promote self-fertilization and/or mating among genetically related individuals even in the dioecious, obligate outcrosser *F. vesiculosus*. In addition, the spatial proximity of the two taxa on local scales may also govern the potential and frequency of hybridization, possibly limiting it to zones of immediate contact of the two species.

To date, molecular markers have not settled the question of the specific status of *F. spiralis* and *F. vesiculosus* in the field, nor their propensity for natural hybridization. The genetic and evolutionary relationships between *F. spiralis* and *F. vesiculosus* could not be resolved using nuclear ribosomal DNA (ITS) markers (Serrão *et al*. 1999). Likewise, species-specific cytoplasmic markers successfully employed in detecting hybridization in another pair of *Fucus* species (*F. serratus*/*F. evanescens*; Coyer *et al*. 2002a, b) have not proved to be diagnostic of *F. spiralis* or *F. vesiculosus* (J. Coyer, personal communication). However, the recent development of polymorphic, nuclear, codominant microsatellite markers in *F. vesiculosus* and *F. spiralis* (Engel *et al*. 2003) may provide the much-needed genetic tools for detecting specific genetic entities as this type of marker has successfully differentiated between species of closely related cross-fertile oaks where ITS and chloroplastic markers have failed (Muir *et al*. 2000).

Ideally, detecting distinct taxon units and/or admixture with species-diagnostic genetic systems, whether they be species-specific markers or characterization of allele frequencies in source (reference) populations, requires identification of pure (e.g. parental) populations. Indeed, it is difficult to make estimates of the degree of admixture when the gene frequencies in parental populations prior to admixture are unknown (Bertorelle & Excoffier 1998; Estoup *et al*. 1999; Falush *et al*. 2003). Identifying pure taxon units in the *spiralis*/*vesiculosus* system presents two major difficulties. First, contrary to classic hybrid zones — zones of contact on the edges of the otherwise allopatric parental species' distributions — taxa co-occur throughout the major part of their ranges. Further, allopatric populations are frequently found in atypical and/or marginal habitats (estuaries, highly exposed open shores) where allele frequencies may not be characteristic. Second, even within a single shore, the reported morphological plasticity makes identification of pure taxon units precarious. The recent development of a model-based Bayesian clustering method (Pritchard *et al*. 2000) circumvents the problem of characterizing taxon units. Based on multilocus genotypes, this statistical tool efficiently groups individuals — without prior knowledge of taxon affiliation — into genetically homogeneous clusters such that Hardy–Weinberg and linkage disequilibria within clusters are minimized. Further, while clusters are being defined, individuals are simultaneously assigned to one or more clusters based on individual admixture proportions.

In light of the contrasting mating systems and the putative potential for interspecific gene flow, we aimed to characterize the identity of presumed *F. spiralis* and *F. vesiculosus* populations, both genetically and sexually. Using five recently developed microsatellite loci (Engel *et al*. 2003),

[1]This taxon is often mistakenly referred to as monoecious (also noted by Ladah *et al*. 2003). In *Fucus*, gametangia (sex organs) are located in conceptacles that are grouped together in apical reproductive regions (receptacles). Thus, in analogy to flowering plants (e.g. see Richards 1997) — with conceptacles corresponding to flowers — in hermaphroditic species, conceptacles are cosexual and in monoecious and dioecious species, conceptacles are unisexual (male or female). In monoecious species, receptacles of a genet may bear male and/or female conceptacles while in dioecious species all conceptacles of a genet are of only one sex.

multilocus genotypes were analysed using a Bayesian clustering method (Pritchard *et al*. 2000) to evaluate the existence of two separate genetic entities, to detect putative admixed individuals and to estimate the frequency of admixture between *F. spiralis* and *F. vesiculosus*. We evaluated the distribution of sexual phenotypes and the effect of mating system on the organization of genetic diversity within populations. To evaluate the influence of local-scale taxon distribution, these analyses were carried out in two contrasting spatial configurations, one in which algae typical of each taxon were contiguous and the other where typical algae were strictly non contiguous.

Materials and methods

Sampling

As accurate determination of the sexual phenotype requires microscopy, sampling of *Fucus vesiculosus* and *Fucus spiralis* was primarily based on consensual general overall whole-individual morphology, typical of each taxon (e.g. short, wide thalli with rounded receptacles for *F. spiralis* and longer, thinner thalli with elliptical receptacles for *F. vesiculosus*) and the presence of vesicles (i.e. paired air bladders), a character associated with *F. vesiculosus*, although absent in some individuals of this species). Insofar as possible, putative *F. vesiculosus* individuals having vesicles were sampled; otherwise, the absence of vesicles was recorded. Taxa were sampled in two sites from two distant regions: Cape Gris-Nez in northern France and Viana do Castelo in northern Portugal. In each site, two stations separated by 200 m were chosen parallel to the waterfront (Fig. 1). Stations showed contrasting distribution patterns: taxa were contiguous (mixed stand at boundaries) at one station ('sympatric' station) while at the other station, taxa were strictly noncontiguous ('parapatric' station). At parapatric stations, taxa were separated, perpendicular to the waterfront, by at least 35 m. At each station, 72 individuals of each taxon were mapped and sampled from an area ranging from 5 to 20 m². For each individual, vegetative tips were excised for DNA extraction. In addition, at least three receptacles were sampled from each individual and freeze dried for storage until determination of sexual phenotype.

Sexual phenotype of each individual was determined by examining conceptacles under a brightfield microscope at 100× magnification. To do so, squash preparations were made from receptacles that had been rehydrated with a 10 min soak in distilled water.

DNA for genotyping was extracted from *c*. 1 mg of dried tissue using the DNeasy™ 96 Plant kit (QIAGEN) and diluted to 1:500. Five loci were amplified in each taxon: *L20*, *L38*, *L58*, *L78* and *L94*. Polymerase chain reaction (PCR) amplification was performed using fluorescent-labelled primers as described in Engel *et al*. (2003). Labeled PCR products were analysed on an automated DNA sequencer (LI-COR 4200™) along with a M13 sequence to estimate allele sizes. Banding patterns were occasionally uninterpretable at the *L78* locus; the *L78* genotype of individuals with this pattern was scored as missing data.

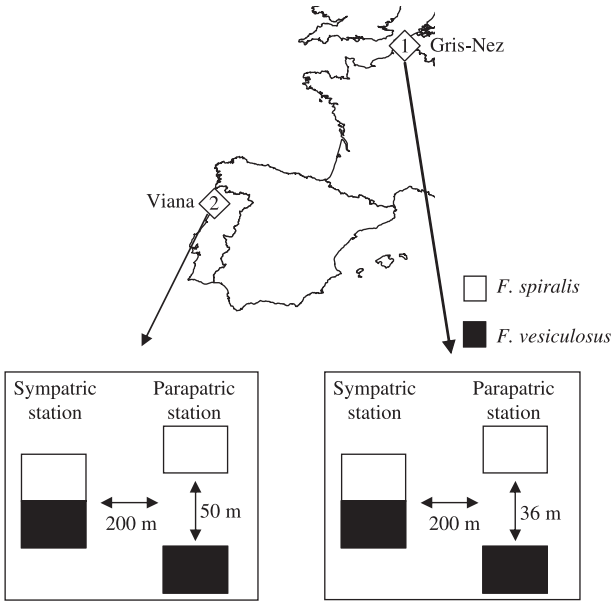

Fig. 1 Sampling scheme of the four study populations at (1) Gris-Nez (northern France) and (2) Viana (northern Portugal). At each site, putative *Fucus spiralis* and *Fucus vesiculosus* individuals were sampled in two different situations; taxa were contiguous in sympatric stations, while taxa were discontinuous in parapatric stations. At each station, 72 individuals of each taxon sample based on general whole-individual morphology were collected.

Genetic analyses

Definition of homogeneous genetic clusters. A model-based genetic admixture analysis, implemented with the software STRUCTURE (Pritchard *et al*. 2000), was used to assign individuals to species and to detect putative hybrids. STRUCTURE uses a Bayesian method that simultaneously identifies clusters (populations) of genetically similar individuals from multilocus genotypes, assigns individuals to these clusters and detects admixed individuals arising from (recent) hybridization and/or introgression of these clusters. For this analysis, we assumed two parental populations (i.e. taxa) ($K = 2$); in this two population case, the mean of the posterior distribution of each individual's admixture coefficient, $\hat{q}_1^{(i)}$, represents the proportion of the i^{th} individual's genotype drawn from population 1. A burn-in of 50 000 repetitions and a run length of 500 000 were used. To avoid potentially confounding geographical structure, the same analysis was carried out for Gris-Nez

and Viana separately. Repeated runs produced results nearly identical to those shown.

Since inbreeding generates (gametic) linkage disequilibrium among loci as well as Hardy–Weinberg disequilibrium, assignment tests are generally inappropriate for endogamous (inbreeding) taxa (see Results); however, provided sufficient genetic differentiation among groups, assignment tests appear to be robust to the mating system (Bonnin *et al*. 2001; see also Cornuet *et al*. 1999; Rosenberg *et al*. 2002; Baudouin *et al*. 2004). Further, although inbreeding reduces the number of genes actually sampled and results in uncertainty in allele frequencies, clustering estimates tend to be unbiased (J. Pritchard, personal communication; see also Baudouin *et al*. 2004). Nevertheless, we tested the validity and coherence of the Bayesian clustering method by comparing individual admixture coefficients with a distance-based measure, which has the advantage of not assuming Hardy–Weinberg or linkage equilibria (e.g. Cornuet *et al*. 1999). We calculated average genetic distances d between each individual i and the members of the two taxon samples ($d^{(i)}_{ves}$, $d^{(i)}_{sp}$, where *ves* indicates the *F. vesiculosus* sample and *sp*, the *F. spiralis* sample) separately for each site using the GENECLASS software package (Piry *et al*. 2004). We used Cavalli-Sforza & Edwards's (1967) chord distances as these showed the best performance in correctly classifying individuals (see Cornuet *et al*. 1999). To obtain a single distance measure per individual, we subtracted the distance to the *spiralis* taxon sample from the distance to the *vesiculosus* taxon sample ($d^{(i)}_{sp-ves} = d^{(i)}_{sp} - d^{(i)}_{ves}$). To compare the distance method with the Bayesian clustering method, we tested the (Spearman rank) correlation of individual distance differences ($d^{(i)}_{sp-ves}$) with individual admixture coefficients, $\hat{q}^{(i)}_1$.

Population genetic structure within and among clusters. Genetic structure analyses were carried out on each of the four clusters defined with the Bayesian assignment method. We calculated the mean number of alleles per locus (A_O) and average expected heterozygosity [nonbiased gene diversity, H_E (Nei 1978)] for both clusters at each station in both sites. To analyse the mating system, fixation indices ($\hat{f}_{IS}$) within each station and cluster (= population) were computed for each locus and heterozygote deficiencies and excesses were tested using 20 000 randomizations of alleles among individuals within each population using the FSTAT software package (Goudet 1995).

We used spatial autocorrelation (Sokal & Oden 1978a, b; Heywood 1991; Epperson & Li 1996) to examine the organization of genetic variation at the within-population level. Under an isolation-by-distance process in a two-dimensional space, the pairwise genetic correlation between individuals is expected to decrease in a roughly linear fashion with the logarithm of the geographical distance (Hardy & Vekemans 1999; Rousset 2000). To estimate pairwise genetic correlation, we used Moran's I statistic, a multiallelic, multilocus relationship coefficient that also has the advantage of being insensitive to selfing rate (see Hardy & Vekemans 1999). Multi- and single-locus pairwise relationship coefficients were calculated for both clusters separately in each station and regressed on pairwise separation distance using the program SPAGEDI (Hardy & Vekemans 2002). The null hypothesis of random spatial distribution of genotypes was rejected if the frequency of the observed or a greater value in the random distribution of I generated from 1000 permutations of individuals among the different localities was < 0.05.

Finally, to elucidate the relationships among clusters from the two sites, we constructed a neighbour-joining (NJ) tree using Cavalli-Sforza & Edwards's (1967) chord distances computed from allele frequency data. Chord distances, not based on a particular mutation model, have been shown to perform well in the reconstruction of phylogenies of closely related taxa (see Goldstein & Pollock 1997). Pairwise distances were computed using the GENDIST program included in the PHYLIP computer package, version 3.5 (Felsenstein 1993). The matrix of pairwise distances was used to construct a phenogram using the NJ algorithm (NEIGHBOR) available in PHYLIP. Confidence levels on tree topology were estimated by the percentage of 1000 bootstraps performed from resampling allele frequencies with SEQBOOT, and compiled using CONSENS, both programs in the PHYLIP package.

Results

Phenotyping and genotyping

All 288 mature individuals sampled as *Fucus vesiculosus* were strictly unisexual at both stations in both sites (Table 1). While the vast majority (> 90%) of the 287 individuals sampled as *Fucus spiralis* showed hermaphroditic conceptacles in the parapatric stations, 29% of individuals from *F. spiralis* sympatric stations were unisexual (Table 1).

Individual genotypes were determined at five microsatellite loci in 566 individuals. All five loci showed substantial variability with 5–10 alleles per locus and a mean value H_E ranging from 0.47 to 0.66 at Gris-Nez and from 0.36 to 0.73 at Viana.

Definition of genetic entities

The posterior distributions of admixture proportions for all individuals were not uniform (Fig. 2). The analysis showed a strong association of individuals sampled in *F. spiralis* taxon samples with one cluster at both sites (proportion of membership, Gris-Nez, $q_1 = 0.929$; Viana $q_1 = 0.761$). The second cluster grouped individuals sampled in *F. vesiculosus* taxon samples (Gris-Nez, $q_2 = 0.942$; Viana,

Table 1 Classification of individuals by sexual phenotypes and clustering results with a summary of genetic diversity statistics for each cluster at each station (GN, Gris-Nez; V, Viana; P, parapatric; S, sympatric) and over all stations and sites

		Taxon sample														
		F. spiralis				*F. vesiculosus*										
Cluster	Station	N_h	N_f	N_m	N_{nd}	N_h	N_f	N_m	N_{nd}	N_T	A	H_E	H_O	Pp (%)	SSA	SSA (> 0.05)
F. spiralis	GN-P	64			5		1			70	1.2	0.003	0.003	0		
	GN-S	53		3	1					57	1.4	0.011	0.011	0		
	V-P	64			2					66	2.0	0.108	0.012	60		
	V-S	33	2	1						36	2.0	0.079	0.000	60		
	Overall	214	2	4	8		1			229	2.6 (0.5)	0.210 (0.116)	0.007 (0.004)	40	1	1
F. vesiculosus	GN-P						27	34	5	66	6.0	0.534	0.534	100		
	GN-S	1	1	1			37	29		69	6.2	0.654	0.548	100		
	V-P			1			29	34	1	65	3.8	0.569	0.454	100		
	V-S	3	5	13			30	28		79	4.4	0.562	0.420	100		
	Overall	4	6	15			123	125	6	279	7.8 (1.5)	0.671 (0.044)	0.491 (0.025)	100	26	10
Intermediate	GN-P	2		1			2	2	1	8	3.6	0.568	0.444	100		
	GN-S	7	2	3			4	2		18	4.0	0.510	0.329	100		
	V-P	4		1			3	5		13	3.8	0.503	0.387	100		
	V-S	3	2	9			3	2		19	3.8	0.587	0.554	100		
	Overall	16	4	14			12	11	1	58	6.2 (1.3)	0.569 (0.070)	0.434 (0.060)	100	—	—

N_h, N_f, N_m, N_{nd}, number of hermaphroditic, female, male and nondetermined individuals, respectively; N_T, total number of assigned individuals; A, mean number of alleles per locus (SE); H_E total expected heterozygosity (SE); H_O, observed heterozygosity (SE); Pp (0.95), percentage of polymorphic loci polymorphism (where most common allele does not exceed 0.95); SSA, number of species-specific (private) alleles; SSA (> 0.05), number of species-specific (private) alleles at a frequency > 0.05.

$q_2 = 0.961$). Thus, in general, the two taxa were split into two different, distinct clusters based only on their genetic make-up and independently of any prior population information.

Individuals showing a $\hat{q}_1^{(i)}$ equal to or greater than 0.90 were assigned to the *spiralis* cluster and those with a $\hat{q}_2^{(i)}$ equal to or greater than 0.90 were assigned to the *vesiculosus* cluster. Although the value of this criterion was somewhat arbitrary (Beaumont *et al*. 2001), it did not greatly affect patterns of assignments of individuals to clusters. Following this classification scheme, individuals that showed a $\hat{q}_1^{(i)}$ between 0.10 and 0.90 were considered to be genetically intermediate to *F. vesiculosus* and *F. spiralis*. These genetically intermediate individuals were more frequent at sympatric stations (12.5% and 14.2% of individuals at Gris-Nez and Viana, respectively) than at parapatric stations (5.6% and 9.0% of the individuals at Gris-Nez and Viana, respectively); this difference was significant when both sites were considered together (Fisher exact tests on 2×2 contingency tables, Gris-Nez, $P = 0.06$; Viana, $P = 0.19$, over both sites, $P = 0.017$).

The first inferred (*F. spiralis*) cluster was generally characterized by hermaphroditic individuals and the second (*F. vesiculosus*) cluster by unisexual individuals with vesicles (Fig. 2). None of the individuals with vesicles were classified as *F. spiralis*. While the majority (47%, Table 1) of the 45 unisexual individuals sampled in *F. spiralis* taxon samples were assigned to the *vesiculosus* cluster, 40% (Table 1) were intermediate to the two clusters. Nonetheless, a few individuals assigned to one of the two clusters showed sexual phenotypes unexpected for that cluster (Table 1). These aberrant phenotypes were observed primarily in the sympatric stations (all sampled as *F. spiralis*), with three unisexual individuals in the *F. spiralis* cluster at both Gris-Nez and Viana and one and three hermaphroditic individuals in the *F. vesiculosus* cluster at Gris-Nez and Viana, respectively. In the parapatric stations, only one unisexual (female) individual, sampled in the supposed *F. vesiculosus* population, was observed in the *F. spiralis* cluster at Gris-Nez. Among the 58 genetically intermediate individuals, the large majority was unisexual (78.1% in Viana, 61.5% in Gris-Nez, Table 1) of which 12 possessed vesicles.

The $d_{sp-ves}^{(i)}$ values were highly (negatively) correlated with $\hat{q}_1^{(i)}$ values in each site (Gris-Nez, Spearman rank correlation, $\rho = -0.965$, $P < 0.001$, $n = 288$; Viana, $\rho = -0.954$, $P < 0.001$, $n = 278$), indicating that the closer an individual was to the *F. spiralis* taxon sample compared to the *F. vesiculosus*

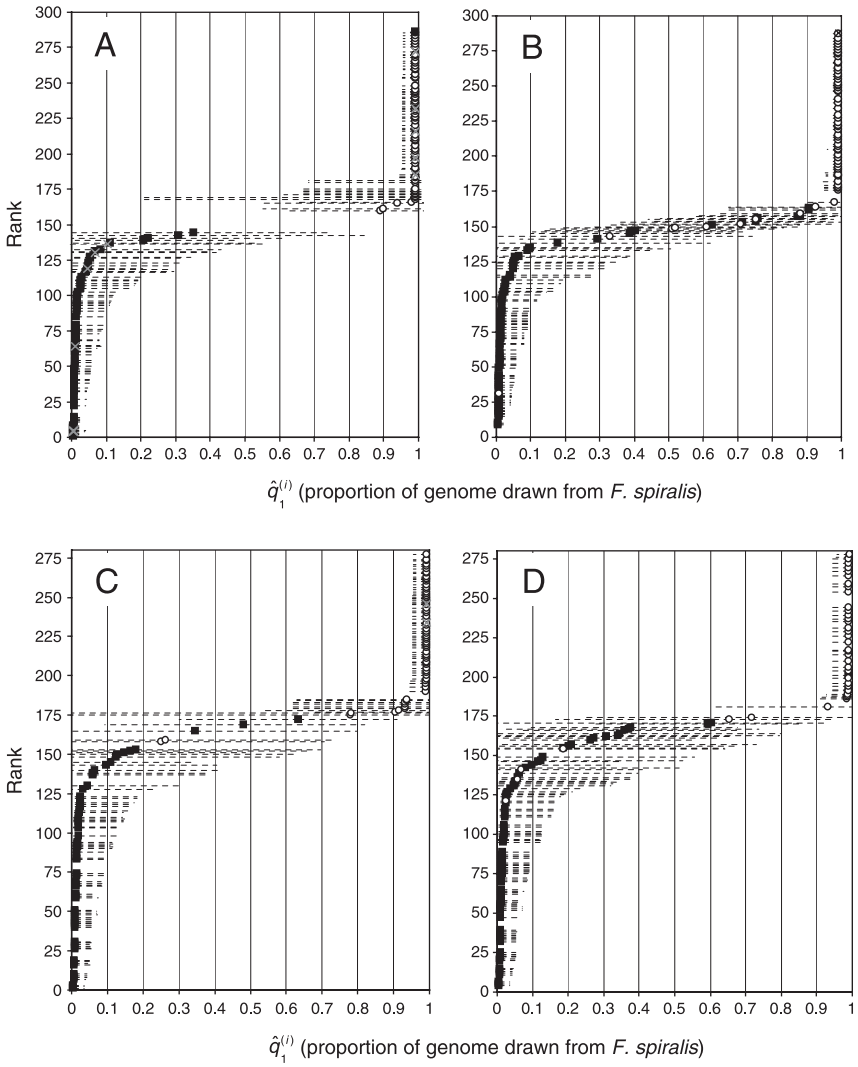

Fig. 2 Bayesian analysis of genetic structure of sampled sexual phenotypes. The distribution of $\hat{q}$ among individuals at the (A) Gris-Nez parapatric (B) Gris-Nez sympatric (C) Viana parapatric and (D) Viana sympatric stations. The values of $\hat{q}$ have been ranked (within each site) and the ranks are plotted against $\hat{q}$, for each station separately. Horizontal lines correspond to the 90% equal-tail posterior probability intervals for each individual. Black squares, unisexual individuals; open circles, hermaphroditic individuals; x, not determined.

sample, the higher its $\hat{q}_1^{(i)}$ values. Further, this significant association was not uniquely due to the extreme values associated with the defined *vesiculosus* and *spiralis* clusters, as the correlation on just the above-defined genetically intermediate individuals (i.e. $0.1 < \hat{q}_1^{(i)} < 0.9$) was also highly significant (Gris-Nez, $\rho = -0.954$, $P < 0.001$, $n = 26$; Viana, $\rho = -0.657$, $P < 0.001$, $n = 32$). The results obtained with STRUCTURE thus appear to be robust to the mating system. Further, in spite of its advantages, the drawbacks of distance methods — low resolution of individual-to-population relationships (e.g. Cornuet *et al*. 1999; Rosenberg *et al*. 2001) and *a priori* definition of (reference) samples — make the clustering method more attractive for detecting genetic entities and admixture.

Within-cluster structure: comparative analysis

Among the three groups, the allele frequency distributions, given by station, varied across loci, with *F. spiralis* populations showing relatively low polymorphism at most loci (Fig. 3). Indeed, two loci, *L58* and *L38*, were monomorphic at the 95% level in all four *F. spiralis* populations and, although polymorphic at Viana, *L94* presented the same most common allele at all stations. In contrast, all loci were highly polymorphic in all *F. vesiculosus* populations. Genetic diversity indices indicate lower genetic diversity in *F. spiralis* than in *F. vesiculosus*, in terms of mean numbers of alleles and expected heterozygosity (both indices, Wilcoxon signed rank test $P = 0.03$, $N = 5$) (Table 1). Genetically intermediate individuals showed diversity indices comparable to those observed in *F. vesiculosus*. All three groups showed deficits in heterozygotes (Table 2).

With the exception of allele 137 at *L78*, all alleles observed in *F. spiralis* clusters were also observed in *F. vesiculosus* clusters, i.e. *F. spiralis* alleles are a subset of *F. vesiculosus* alleles. In contrast, 26 private alleles were observed in *F. vesiculosus* clusters. However, less than half (42%) of these presented overall frequencies greater than 0.05 (Table 1).

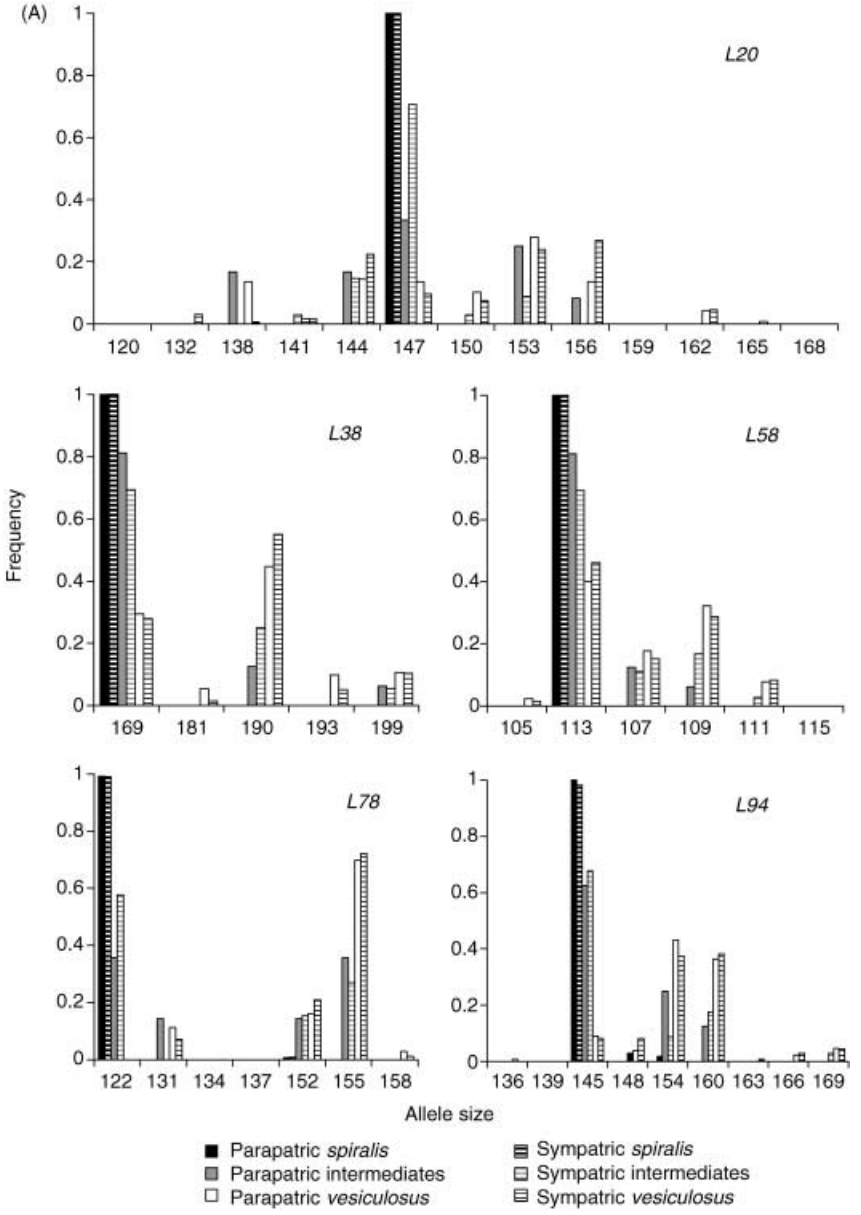

Fig. 3 Allele frequencies at the five microsatellite loci for all three individual classifications *Fucus spiralis* (black), *Fucus vesiculosus* (white) and intermediates (grey) at parapatric (solid bars) and sympatric (striped bars) stations in Gris-Nez (A) and Viana (B).

Table 2 Single- and multilocus $\hat{f}_{IS}$ values for each cluster at each station. Abbreviations as in Table 1

	F. spiralis clusters				*F. vesiculosus* clusters			
Locus	GN-P	GN-S	V-P	V-S	GN-P	GN-S	V-P	V-S
L20	—	—	1.000***	1.000***	0.316***	0.207**	0.575***	0.424***
L38	—	—	—	—	0.238*	0.131	0.147	0.170
L58	—	—	—	—	0.236*	0.220	0.271	0.330***
L78	—	—	0.819	1.000**	0.093	0.260	−0.106	0.105
L94	—	—	0.904***	1.000***	0.124	0.016	0.108	0.249
Multilocus	—	—	0.886***	1.000***	0.214***	0.162***	0.203***	0.254***

—, monomorphic locus, $\hat{f}_{IS}$ undefined.
*$P < 0.05$, ***$P < 0.001$ where P is the probability associated with permutation tests.

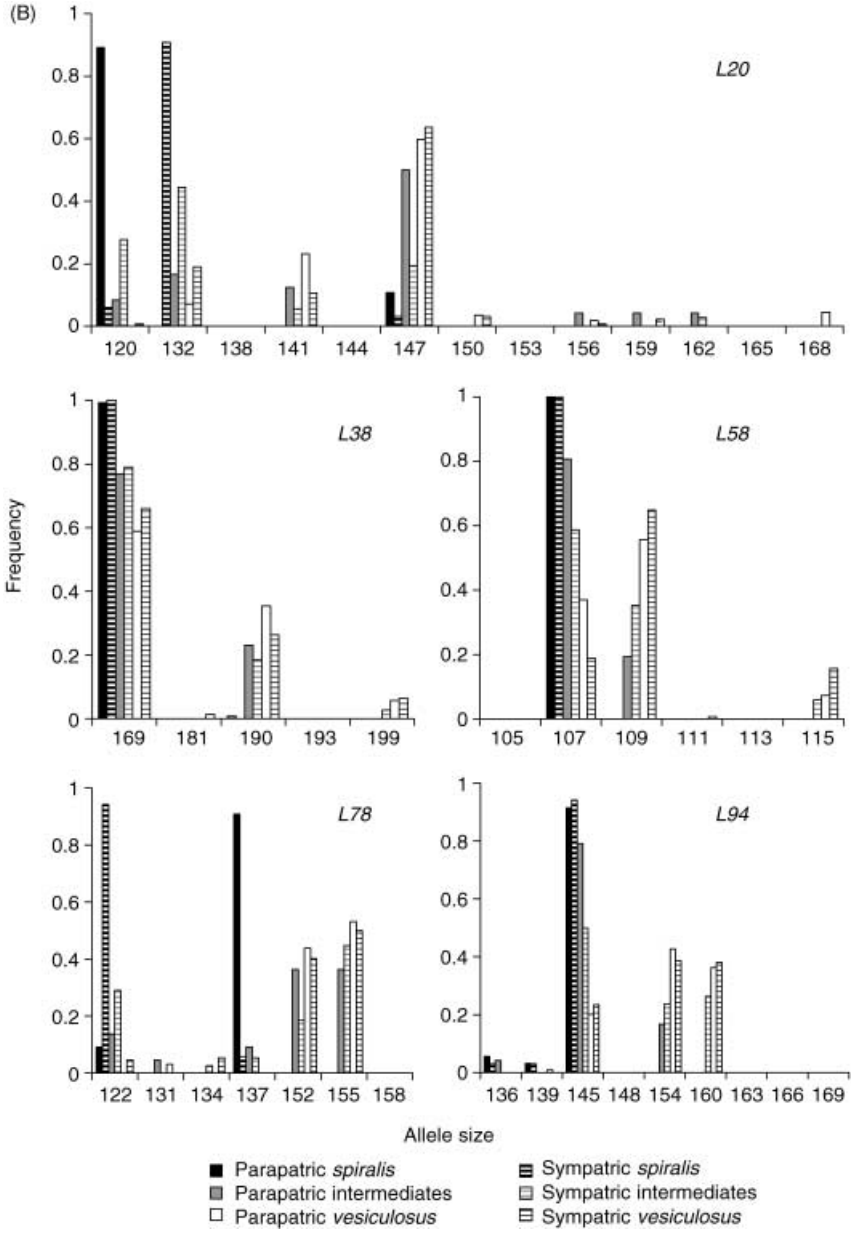

Fig. 3 *Continued*

In addition to the lone private allele specific to *F. spiralis* clusters, two other alleles could be described as 'taxon-associated' (see Kumar *et al*. 2003): allele 120 at *L20* and allele 122 at *L78*, although shared with *F. vesiculosus* clusters were rare (< 0.05) in this latter taxon. By the same criteria, three alleles present in both taxa could be considered '*vesiculosus*-associated' (allele 190 at *L38*, allele 152 at *L78* and allele 154 at *L94*). Inspection of the distribution of alleles in each of the *a posteriori* clusters shows that none of the alleles were diagnostic (i.e. there were no fixed differences between the two taxa); the detection of two conspicuous genetic entities was largely based on differences in allele frequencies between the two clusters.

Multilocus $\hat{f}_{IS}$ values indicated high heterozygote deficits and were highly significant for both clusters at both stations, with the exception of Gris-Nez *F. spiralis* clusters where extremely low polymorphism precluded analysis of the mating system. Heterozygote deficits were much more severe in *F. spiralis* clusters (global $\hat{f}_{IS}$, 0.850) than in *F. vesiculosus* clusters (global $\hat{f}_{IS}$, 0.204). Single-locus estimates were generally positive, but only the most polymorphic locus, *L20*, was consistently significant across both stations at both sites.

In *F. spiralis* clusters, fine-scale spatial structure could only be analysed at Viana, where genetic relationship coefficients were consistently negatively correlated with

Table 3 Slopes of autocorrelograms for each locus and overall loci. Abbreviations as in Table 1

	F. spiralis clusters		*F. vesiculosus* clusters			
Locus	V-P	V-S	GN-P	GN-S	V-P	V-S
L20	−0.117***	−0.048	0.019	0.004	−0.024	0.021
L38	—	—	0.019	−0.012	0.014	−0.008
L58	—	—	−0.024	0.003	0.009	0.006
L78	−0.109***	−0.064	0.023	−0.005	−0.012	−0.031
L94	0.004	−0.043	0.010	−0.009	−0.003	−0.023
Multilocus	−0.078***	−0.046	0.007	−0.004	−0.002	−0.010

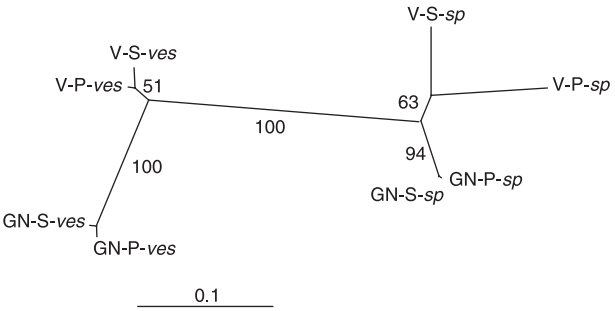

Fig. 4 Neighbour-joining tree based on Cavalli-Sforza & Edwards's (1967) chord distances. Percentages indicate robustness of given branch out of 1000 bootstrap permutations of allele frequencies. GN, Gris-Nez; V, Viana; P, parapatric station; S, sympatric station; *sp*, *F. spiralis* cluster; *ves*, *F. vesiculosus* cluster.

the logarithm of distance. Although only significant at the parapatric station, the slopes are of the same order of magnitude, suggesting that the absence of heterozygotes at the sympatric station reduced statistical power. In contrast, in *F. vesiculosus* clusters, none of the single-locus or multilocus regression slopes, roughly an order of magnitude lower, were significantly different from zero (Table 3), indicating that genotypes were generally randomly distributed within populations, i.e. within an area of 5–20 m², in this taxon.

Among-cluster structure

Finally, the NJ tree revealed two distinct clades clearly separating the *spiralis* and *vesiculosus* clusters from each station based on taxon identity rather than on geographical location (Fig. 4). Furthermore, relationships were poorly supported within each taxon cluster at Viana, while at Gris-Nez conspecific individuals from both stations clearly shared an exclusive common ancestor, less distant than any possible common ancestor to the conspecific individuals sampled at Viana.

Discussion

Genetic evidence for distinct species in the field

Our analysis, based on five-locus microsatellite genotypes without using any prior information on taxon affiliation, allowed us to identify two distinct genetic entities in the *Fucus spiralis/vesiculosus* system: the distribution of individual admixture proportions was highly asymmetrical and the probability limits for most individuals assigned to one of the two clusters were close to 0 or 1 at both sites (Fig. 2). One cluster grouped a vast majority of unisexual individuals bearing vesicles, typical of the described species *Fucus vesiculosus* and the other cluster was characterized by hermaphroditic individuals, traditionally ascribed to *Fucus spiralis*. In addition, the vast majority (85%) of individuals were assigned to the taxon they were sampled as (Table 1), indicating that field-based overall whole-individual morphology was a good indicator of species. Furthermore, these appear to be cohesive species units as the NJ tree (Fig. 4) clearly groups clusters according to taxon identity rather than to geographical location. In addition, an analysis of genetic admixture combining both sites resulted in 94.7% of individuals assigned to the same taxon as in the separate analysis for each site (results not shown). This result indicates that general (overall) differentiation among taxa was the major component of the local (site) differentiation, again demonstrating the cohesiveness of the genetic entities. Nonetheless, our results caution against using sexual phenotypes and/or vesicles for species identification: no mating system was strictly exclusive of either cluster and vesicles were observed on individuals not assigned to either cluster. Indeed, misleading, incorrect assignment of parental or hybrid status of individuals was a caveat in previous work on hybridization in this *Fucus* system which relied upon *a priori* phenotypic criteria to assign individuals to a specific taxon (Burrows & Lodge 1951; Pérez-Ruzafa *et al*. 1993; Scott & Hardy 1994).

Although unisexual individuals were typical of lower-shore positions of the *F. vesiculosus* taxon samples and hermaphrodites of the high-shore positions of *F. spiralis* taxon samples, migrants of both clusters were detected in high- and low-shore positions. However, 21 (all unisexual) *F. vesiculosus* migrants (i.e. individuals collected in the *F. spiralis* taxon sample but assigned to the *F. vesiculosus* cluster) were detected in *F. spiralis* high-shore positions compared to only one (female) *F. spiralis* migrant (Table 1) found at a *F. vesiculosus* mid-shore position. Although the vast majority (20/21) of these migrants were found in sympatric stations, such a pattern would be consistent with greater dispersal of *F. vesiculosus* gametes (or zygotes) compared to *F. spiralis* gametes (or zygotes) (see next section). However, the presence of *F. vesiculosus* in typical *F. spiralis* positions and the complete absence of

hermaphrodites in lower-shore *vesiculosus* taxon samples raise questions as to the mechanics of the zonation of the two species (cf. Karez & Chapman 1998).

Contrasting breeding systems revealed in the hermaphroditic and dioecious taxa

Both clusters showed highly significant $\hat{f}_{IS}$ values, although *spiralis* clusters showed values roughly four times greater than those found in *vesiculosus* clusters. *Fucus vesiculosus* clusters showed values that are characteristic of endogamous species, while the high values (and reduced levels of genetic variation) in the hermaphroditic *F. spiralis* clusters are typical of selfing species (Hamrick & Godt 1997; Charlesworth & Wright 2001; see also, e.g. Viard *et al*. 1996; Awadalla & Ritland 1997; Bonnin *et al*. 2001).

As selfing is not possible in species with separated sexes, inbreeding may arise from sexual inconstancy, limited gamete dispersal or Wahlund effect in *F. vesiculosus*. First, four hermaphrodites were detected in *F. vesiculosus* clusters; sexual inconstancy may thus contribute to heterozygote deficits if hermaphrodites tend to self-fertilize (see below). Second, biparental inbreeding implies that siblings mate more often than at random; this may be due to limited gamete dispersal or to phenological effects. However, spatial autocorrelation analysis showed that genotypes were distributed randomly in space, suggesting that gamete dispersal is not limited on scales of less than 5–7 m, contrary to expectations from dispersal and recruitment studies with fucoid algae (e.g. Serrão *et al*. 1997; Arrontes 2002). Although the majority of the eggs appear to settle within 2 m of the mother alga, some eggs and sperm are capable of dispersing beyond the maximal sampled distance of 2–6 m (Serrão *et al*. 1997; Dudgeon *et al*. 2001); further, drifting fertile thalli have been recorded to produce recruits after dispersing for hundreds of metres (C. Faustino, E. Serrão, G. Pearson, Universidade do Algarve, unpublished). The lack of fine-scale spatial structure despite significant inbreeding may indicate that related individuals may preferentially mate if, for example, they are more often simultaneously reproductively mature than unrelated individuals. Finally, temporal Wahlund effects may explain positive $\hat{f}_{IS}$ values: deficits in heterozygotes may arise in (i) an age-structured population whereby (successive) cohorts of recruits are genetically differentiated and/or (ii) when phenological differences impose different reproductive periods for different subsets of individuals. Temporal Wahlund effects were also evoked for mild heterozygote deficiencies associated with high genetic diversity and an absence of fine-scale structure observed in dioecious *F. serratus* (Coyer *et al*. 2003).

Although the selfing rate was not measured directly (Ritland & El-Kassaby 1985), the great heterozygote deficiencies in the hermaphroditic taxon *F. spiralis* can only be explained by high rates of selfing. The very low polymorphism at the microsatellite loci used here means that the estimation of $\hat{f}_{IS}$ — possible in only two populations — was based on only three loci (Table 2**)**, of which only two were polymorphic over all populations (Table 1). For these two species-wide polymorphic loci, as in other selfing species (plants, Bonnin *et al*. 2001; Charlesworth & Pannell 2001; fern, Vitalis *et al*. 2002; mollusk, Viard *et al*. 1996), populations were fixed (or nearly so) for alternate alleles. Selfing in *F. spiralis* thus appears to be prevalent *in natura* despite dispersal of both male and female gametes before syngamy. Fine-scale spatial structure was detected in polymorphic populations of *F. spiralis*; this pattern is consistent with low gamete (and zygote) dispersal. Self-fertilization may also be facilitated by phenological differences whereby only self-sperm is available to fuse with an individual's eggs. Other mechanisms may promote self-fertilization: the simultaneous release of gametangia (oogonia and antheridia) and the possibility of sperm (rapidly liberated from antheridia) to penetrate the inner layer of oogonia, before release of the eight eggs therein (Müller & Gassman 1984).

Genetic intermediacy and species integrity

Although the two clusters are clearly differentiated genetic entities and show strongly contrasting patterns of genetic structure, allele ranges (Fig. 3) and distributions of individual admixture proportions (Fig. 2) suggest that the *F. vesiculosus* and *F. spiralis* clusters possess a certain degree of genetic continuity (Fig. 2). First, inspection of the distribution of alleles in each of the *a posteriori* clusters shows that none of the alleles were diagnostic (i.e. there were no fixed differences between the two taxa). This indicates that the detection of two conspicuous genetic entities was largely based on differences in allele frequencies between the two clusters. Furthermore, the vast majority of alleles found in the *F. spiralis* clusters were a subset of those found in the *vesiculosus* clusters. Only one locus, *L78*, showed species-specific/taxon-associated alleles for both taxa; in the four other loci, most private alleles were detected in the *vesiculosus* cluster. Second, our analysis revealed 58 (overall mean, 10.2%) individuals which were genetically intermediate to both taxon clusters. However, the existence of many private alleles generally indicates little gene flow between two genetic entities (Slatkin 1985). Likewise, other studies using microsatellites showing substantial numbers of private alleles generally observe fewer individuals with presumably hybrid ancestry (e.g. deer, Goodman *et al*. 1999; wildcats, Randi *et al*. 2001; oaks, Craft *et al*. 2002; canids, Randi & Lucchini 2002). This apparent discrepancy between strong differentiation and presence of substantial numbers of genetically intermediate individuals can be explained by recent divergence of two taxa with retention of ancestral polymorphism or asymmetrical introgressive

gene flow from *F. spiralis* to *F. vesiculosus*. In both cases, the contrasting breeding systems are probably responsible for the observed asymmetry in species-specific alleles and high degree of genetic differentiation. The predominantly selfing regime of *F. spiralis* lowers the genetic effective population size and genetic diversity (Pollak 1987), enhancing divergence from its outcrossing relative.

Given the cohesion of the taxa across their geographical range (Fig. 4) and (near) fixation in the *spiralis* clusters for the same allele at three out of five loci, divergence between *F. spiralis* and *F. vesiculosus* must have occurred before the range expansion of both taxa through the English Channel to the North Sea after the Last Glacial Maximum. Indeed, Viana, south of the hypothesized polar front located near the northern coast of the Iberian Peninsula (CLIMAP Project Members 1994; Quaternary Environments Network 1995), may be part of vestigial refugia. Gris-Nez, on the other hand, located at the boundary between the Channel and the North Sea, probably opened up only 5000 years BP (Quaternary Environments Network 1995). Whatever the scenario of recolonization of the English Channel, it is highly unlikely that same allele be (nearly) fixed in *F. spiralis* at both sites and populations at three loci (i.e. *L38*, *L58* and *L94*; Fig. 3). The coincident monomorphism at these loci suggests that genetically intermediate individuals are the products of interspecific gene flow that has occurred after divergence of the two taxa.

Compelling spatial evidence points to interspecific hybridization as the origin of genetically intermediate individuals: significantly more intermediates were observed in sympatric stations than in parapatric stations (i.e. 13% vs. 7%). As microsatellite loci are presumably selectively neutral, this trend observed in both sites cannot be explained by shared ancestral polymorphisms. The nearly twofold difference suggests that, on a very local scale, contiguous distributions of *F. spiralis* and *F. vesiculosus* facilitate — but do not confine — interspecific gene flow (cf. Dodd & Afzal-Rafii 2004). Although rarer in noncontiguous distributions, the observation of intermediates in both taxon samples in parapatry suggests that heterospecific gamete dispersal occurs both upshore and downshore. In contrast, a study on natural hybridization between *F. serratus* and *F. evanescens* demonstrated that interspecific gene flow was solely restricted to mixed stands (Coyer *et al*. 2002a). Barriers to interspecific gene flow may be weaker in the *vesiculosus*/*spiralis* system than in the *serratus*/*evanescens* system, where successful hybridization perhaps occurs only in situations of regular, repeated contact between heterospecific gametes. Intriguingly, in a recent study of fucoids in a New England estuary (Wallace *et al*. 2004), *Fucus* individuals showing a 'muscoides-like' form — claimed to be hybrids between *F. spiralis* and *F. vesiculosus* — were less frequent in sympatric situations: only 11% of the muscoides-like individuals were sampled in sites where both putative parental species were present.

The occurrence of asymmetric introgression from *F. spiralis* to *F. vesiculosus* would be consistent with species' mating and breeding systems. Given the low sperm production (i.e. sperm:egg ratio) in *F. spiralis* (Vernet & Harper 1980) typical of selfing species (see Williams 1975), *F. spiralis* sperm cannot compete with *vesiculosus* sperm. Any interspecific gene flow is thus most likely the product of rare, unfertilized *F. spiralis* eggs crossed with *F. vesiculosus* sperm. In subsequent crosses, while hermaphroditic hybrid individuals probably self (and thereby do not backcross), any unfertilized unisexual hybrid eggs are more likely to be fertilized by *vesiculosus* sperm and, if competitive, unisexual hybrid sperm may fertilize *vesiculosus* eggs. Indeed, frequent *F. spiralis* alleles were observed in all four *F. vesiculosus* populations at at least four loci (Fig. 3) while the low frequency of *F. vesiculosus* alleles in *F. spiralis* clusters discounts the possibility of substantial introgression towards *F. spiralis* clusters. Likewise, the large range of q values (Fig. 2) and intermediates' allele frequencies and genetic variation (Fig. 3, Table 1) is not consistent with the occurrence of only first-generation hybrids, suggesting that putative *F. spiralis*/*F. vesiculosus* hybrids are fertile and persist beyond the initial hybridization event(s). This pattern contrasts sharply with the study on natural hybridization between *F. serratus* and *F. evanescens* where all hybrids unequivocally appeared to be from the F_1 generation (Coyer *et al*. 2002a). Similarly, Wallace *et al*. (2004) assert — albeit without adequately considering the genetic variability within the putative parental species — that the *muscoides*-like *Fucus* in a New England salt marsh was composed mainly of F_1 hybrids between *F. vesiculosus* and *F. spiralis*. In fact, the actual hybrid status of these *muscoides*-like individuals is questionable as their genetic signatures were similar to some *F. spiralis* individuals.

The major caveat in this study was the absence of diagnostic alleles. In spruces and oaks, groups known for their high propensity for interspecific hybridization, genome-wide surveys (e.g. RAPDs) revealed only ≤ 1% of markers that proved to be diagnostic (Howard *et al*. 1997; Perron & Bousquet 1997). Indeed, where interspecific gene flow is (or was recently) possible, only loci involved in adaptive divergence, or those tightly linked to these regions, may show fixed differences in parental taxa due to negative selection (e.g. Barton 2001; Martinsen *et al*. 2001; Machado *et al*. 2002). Increasing the number of loci using the Bayesian approach employed here would not only increase the power but also may reveal diagnostic markers of the two taxa of this species complex. For instance, four new microsatellite loci developed for *F. spiralis* showed some low-frequency private alleles in this species when compared with *F. vesiculosus* at one location (Wallace *et al*. 2004). Furthermore, according to the asymmetrical introgressive hybridization scenario, assuming maternal-inheritance of organelles (Brawley *et al*. 1976; Coyer *et al*. 2002b), *F. spiralis*

cytoplasm would be present in a *F. vesiculosus* nuclear background. Using cytoplasmic markers Coyer *et al*. (2002a) demonstrated that all detected field hybrids between hermaphroditic *F. evanescens* and dioecious *F. serratus* were crosses between *F. evanscens* eggs and the *F. serratus* sperm. Clearly, polymorphic cytoplasmic markers need to be developed in *F. spiralis* and *F. vesiculosus* to test the occurrence and direction of interspecific gene flow (see Coyer *et al*. 2002a).

Conclusion

In light of the contrasting mating systems that present *Fucus spiralis* and *Fucus vesiculosus*, whether recently separated and/or recently introgressive, the divergent breeding systems probably contribute to species integrity. Selfing increases premating isolation, and thereby creates a barrier to complete mixing of the two taxa. This reproductive isolation may be driven by divergent (exogenous) selective pressures in different habitats (Arnold 1997; see also, e.g. Dorken *et al*. 2002; Dorken & Barrett 2003). As an upper-shore species, *F. spiralis* occupies an extreme environment, undergoing severe desiccation stress (e.g. Davison & Pearson 1996) that may actually favour self-fertilization. Self-fertilization maintains favourable co-adapted gene combinations by reducing (heterologous) recombination (Stebbins 1950). Furthermore, self-fertilization ensures reproduction when sperm may be limiting and outcrossing precarious (Baker 1955; see Vernet & Harper 1980). Nonetheless, our study also detected a homogeneous *F. vesiculosus* cluster, the mechanism by which *F. vesiculosus* remains a cohesive unit and — by the same token, by which *F. spiralis* is excluded from lower-shore positions — remains to be elucidated.

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Carolyn Engel and Claire Daguin collaborated on this study when both were post-doctoral associates in Ester Serrão's lab. Carolyn Engel's research continues to focus on the evolution, genetics and ecology of seaweed species. Claire Daguin is now in charge of the development of genetic markers for marine invertebrate and seaweed species. Ester Serrão leads a research group that is primarily interested in the reproductive ecology, adaptation and population genetics of marine macrophytes.
