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Implications of mating system for genetic diversity of sister algal species: *Fucus spiralis* and *Fucus vesiculosus* (Heterokontophyta, Phaeophyceae)

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The implications of mating system for genetic diversity were assessed in the sister species *Fucus spiralis* and *Fucus vesiculosus* using a combination of ten microsatellite markers. Five new microsatellite markers specific for *F. spiralis* were developed in order to increase marker resolution and complement the results (i.e. mating system and genetic diversity extended to a larger geographic scale) acquired using five microsatellite loci previously developed from a mixed fucoid seaweed DNA library that excluded *F. spiralis*. Low genetic diversities observed at the population and species level in *F. spiralis* using the five new *F. spiralis*-specific loci described here were consistent with the results obtained previously with non-specific microsatellite loci. Results revealed that selfing is characteristic in *F. spiralis* across its latitudinal distribution along the Iberian and French Atlantic coasts. Higher levels of within-population genetic diversity were observed in the outcrossing species *F. vesiculosus*, decreasing towards the southern distributional range of the species. Some cases of significant biparental inbreeding in this species are indicative of short gamete dispersal or mating of spatially or temporally structured populations. In contrast to within-population diversities, higher total genetic diversity among populations was observed in the hermaphroditic species in comparison to the dioecious *F. vesiculosus*.

Key words: dioecious, genetic diversity, hermaphrodite, marine reproductive ecology, mating system, microsatellites, self-fertilization

Introduction

Mating systems have important evolutionary consequences for the distribution of genetic diversity and gene flow within and between populations. The mating system of organisms such as plants and algae can vary from outcrossing to self-fertilization, enabling a species to respond to changing conditions, and can function as the driver for reproductive isolation and eventually for speciation (Charlesworth, 1992; Charlesworth & Charlesworth, 1995; Holsinger, 2000). While the maintenance of genetic diversity and avoidance of inbreeding depression are important advantages for outcrossing species (Maynard Smith, 1978; Charlesworth, 1980), reproductive assurance and colonizing capacity (Baker's rule) may be advantageous for self-fertilizing species (Pannell & Barrett, 1998, 2001). However, high selfing rates in hermaphroditic/monoecious species reduce intrapopulation genetic variation, effective population sizes and gamete dispersal in comparison with obligatory outcrossing dioecious species (Hamrick & Godt, 1997; Holsinger, 2000). Mating systems thus have implications that are central to our understanding of species distribution and evolution.

Sister species with similar geographic ranges but contrasting reproductive modes provide unique opportunities for understanding the effects of mating system on the ecology and evolution of populations. Excellent models for this theme are the morphologically similar and phylogenetically closely related (Serrão *et al.*, 1999; Coyer *et al.*, 2006a) brown algal species *Fucus spiralis* L., in which individuals have both sexes in every reproductive organ (hermaphroditic) and *F. vesiculosus* L., in which individuals are unisexual (dioecious). The two species are dominant producers on temperate rocky shores of the northern hemisphere, typically distributed in the mid-upper intertidal zone, with *F. spiralis* occurring on

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average higher than *F. vesiculosus*. The two taxa have broad areas of sympatry across their distribution, and their hybridization has been suggested by laboratory crosses between the two species (Kniep, 1925) as well as the occurrence in natural populations of intermediate phenotypes (Burrows & Lodge, 1951; Scott & Hardy, 1994; Billard *et al*., 2005*a*) and genotypes (Wallace *et al*., 2004, 2006; Engel *et al*., 2005, 2006, Coyer *et al*., 2006*b*). However, at their south-eastern distributional limit (southwest Iberia–Morocco), their distribution is allopatric; the hermaphroditic *F. spiralis* occurs on the open coast whereas the dioecious *F. vesiculosus* is present only in isolated protected areas such as estuaries and coastal lagoons. Besides their differences in breeding strategy, these two species differ also in stress-resistance and competitive ability (reviewed by Chapman, 1995 and Davison & Pearson, 1996); any or all of these factors are likely to be important in determining their contrasting distribution at their southern limit (Ladah *et al*., 2003), where rocky shores are highly exposed to wave action and desiccation stress.

In *Fucus* species, fertilization is external, limiting the potential for self-fertilization in the hermaphroditic species. Nevertheless, self-fertilization in *F. spiralis* may sometimes occur before gamete release (Müller & Gassmann, 1985). In the dioecious species *F. vesiculosus*, the occurrence of unisexual individuals only, with no gender switches known to occur during lifetime, makes outcrossing obligatory. Fertilization success is estimated to be high in both *F. spiralis* and *F. vesiculosus* (Serrão *et al*., 1996; Berndt *et al*., 2002; Ladah *et al*., 2003). Both species show synchronization of gamete release within species (Serrão *et al*., 1996; Berndt *et al*., 2002; Ladah *et al*., 2003) and limited spatial dispersal of female gametes (Chapman, 1995; Serrão *et al*., 1997) that may lead to local genetic structuring within *Fucus* populations and effective self-fertilization in the self-compatible species. However, in the dioecious *F. vesiculosus*, spatial autocorrelation of microsatellite alleles indicates that global dispersal within populations may be much more extensive than such localized scales estimated directly from female gametes only (Engel *et al*., 2005). In *F. spiralis*, fine-scale spatial structure was detected, and selfing appeared to be the prevalent mode of reproduction (Engel *et al*., 2005).

Unfortunately, the very low degree of polymorphism in *F. spiralis* at the five microsatellite loci used by Engel *et al*. (2005) permitted the estimation of inbreeding coefficients at only two *F. spiralis* sampling sites and this was based on only three loci, of which only two were polymorphic over all sampling sites. The five microsatellite markers were mostly tri-nucleotide repeats that, coupled with *F. spiralis* reproductive mode, may explain their low polymorphism (Chakraborty *et al*., 1997; Ellegren, 2000). Furthermore, these microsatellites were developed from the fucoid seaweeds *F. vesiculosus*, *F. serratus* and *Ascophyllum nodosum* (Engel *et al*., 2003), and excluded *F. spiralis*. A decrease of microsatellite polymorphism with increasing evolutionary distance from the species in which the microsatellites have been developed, has been reported in other species (Rico *et al*., 1996; Steinkellner *et al*., 1997). Although unlikely between closely related species, it is possible that the cross-amplification of these markers in *F. spiralis* resulted in very low polymorphism or monomorphism at the species level. Null alleles are a less probable hypothesis since DNA from almost all individuals was successfully amplified at all loci in *F. spiralis* (Engel *et al*., 2005).

Five new polymorphic microsatellite markers were thus developed specifically for *F. spiralis* in order to increase the resolution of the markers in this species and to complement the results (i.e. mating system and genetic diversity extended to a larger geographic scale) acquired using the five microsatellite loci previously developed by Engel *et al*. (2003, 2005). The combination of the 10 microsatellite loci was then used to assess the implications of mating system for genetic diversity within and among populations in the sister species *F. spiralis* and *F. vesiculosus*, in populations from the centre to the southern limit of distribution of the two species.

Materials and methods

Isolation of microsatellite loci for F. spiralis

Genomic DNA was extracted using the CTAB protocol (Doyle & Doyle, 1987) from six *F. spiralis* individuals from different locations (i.e. Viana, Ribeira d'Ilhas, Almograve [Portugal], and Bude [England]). Pooled DNAs (10 μg) were then digested by *AfaI* (Promega) and ligated to adaptors Rsa21 (5′-CTCTTGCTTACGCGT GGACTA-3′) and Rsa25 (5′-TAGTCCACGCGTAAC GAAGAGCACA-3′). Digested DNAs were purified with High Pure PCR product purification Kit (Roche) and asymmetrically amplified using Rsa21. Purified PCR products were simultaneously enriched by hybridization to CT_{15} 5′-biotinylated repeat probes attached to streptavidin-coated Magnesphere® paramagnetic beads (Promega). Two rounds of enrichments were performed. Enriched fractions were amplified, purified and ligated into pGEM®-T easy vector (Promega) and transformed into *Escherichia coli* JM109 competent cells (Promega). Recombinant clones were selected and insert sizes determined by standard PCR with universal plasmid primers. PCR products were dot-blotted on a nylon membrane and screened by hybridization with

^{32}P-labelled CT_{15} probes. Autoradiography revealed 181 positive clones from a total of 384. Twenty clones were sent for sequencing (Macrogen, Korea), of which 18 contained microsatellite sequences, and primer pairs were manually designed for these (Table 1).

DNA from eight individuals of *F. spiralis* and *F. vesiculosus* (total 16) that displayed several alleles or heterozygote genotypes at loci L20, L58, L94, L38 and L78 (the amplification of these loci is described in the next section) were genotyped to assess polymorphism of the *F. spiralis*-microsatellite loci. Amplifications were performed in 10 µl of reaction mixture containing about 20–200 ng DNA, 0.18 mM each of dC, dG and dTTPs, 2 mM $MgCl_2$, 1 × Taq DNA polymerase Buffer, 7 µM of dATPs, 10 µM of ^{35}S dATP, 1 µM of forward and reverse primers and 0.1 unit of Taq DNA polymerase (Invitrogen). Different PCR conditions were used for different primers (Table 1). Alleles were separated on a denaturing polyacrylamide gel (6%) in 1 × TBE for 1.5 h at 60 W and visualized by autoradiography. All 18 primer pairs amplified DNA fragments of expected sizes in both species. In total, five new polymorphic loci in *F. spiralis* were identified (Fsp1 to Fsp5, Table 1) and their uniqueness was assessed by sequence alignment with all *Fucus* species' microsatellite sequences available in Genbank (Coyer *et al*., 2002; Engel *et al*., 2003; Wallace *et al*., 2004).

Sample collection and genotyping

Individuals of *F. spiralis* and *F. vesiculosus* were collected at low tide in 2001 and 2002. For each species, 24 individuals per quadrat (1 m^2) were randomly sampled in seven locations distributed along the French, Spanish and Portuguese Atlantic coasts (Fig. 1). From these locations, 163 and 168 individuals of *F. spiralis* and *F. vesiculosus*, respectively, were genotyped using both the five microsatellite loci developed in this study and five microsatellite loci previously developed by Engel *et al*. (2003) (L20, L58, L94, L38, L78). Genomic DNAs of *F. spiralis* and *F. vesiculosus* were extracted from about 4 mg of dried vegetative tips using DNeasy™ Plant Mini Kit (Qiagen). PCR amplifications of the ten microsatellite loci were performed in 10 µl of reaction mixtures containing about 20–200 ng DNA, 0.2 mM of each dNTP, 1.5–2.5 mM $MgCl_2$, 1 × Taq DNA polymerase Buffer, 0.3 µM of fluorescently labelled forward primer, 1 µM of reverse primer and 0.5–1 unit Taq DNA polymerase (Invitrogen). PCR conditions are presented in Table 1. Alleles were separated on denaturing polyacrylamide gels (5%) in 1 × TBE for 2.5 h on an ABI 377 automated DNA sequencer.

Data analyses

Microsatellite data were checked for null alleles using the inbreeding corrected null allele estimator of the Excel macro for rare null alleles implemented with the software MICRO-CHECKER (van Oosterhout *et al*., 2003, 2006). For a given locus, the inbreeding coefficient was estimated from our combination of microsatellite loci excluding the locus analysed and locus Fsp3 (see Results for details).

The resolution of the markers was assessed by comparing the levels of genetic diversity within species among combinations of loci as well as analysing the distribution of the proportion of distinct genotypes detected by these markers in the sample (genotypic richness) for all possible combinations of any number of microsatellite loci following the approach of Arnaud-Haond *et al*. (2005). This distribution illustrates our ability to characterize each sexually produced individual by a unique multilocus genotype. Genotypic richness across locations was estimated following Dorken & Eckert (2001) as $R=(G-1)/(N-1)$, where G is the number of multilocus genotypes and N is the sample size. Genotypic richness for all possible combinations of loci was calculated using the program Gencount (available upon request from F. Alberto, University of Algarve, Faro, Portugal) and their distribution described as medians and error bars showing maximum and minimum values. The difference of mean genetic diversity parameters between tri- and dinucleotide loci was tested using a two-tailed paired *t*-test across loci using the program SPSS (SPSS Inc., 1989–2002).

Allele frequencies were calculated for each species at each locus and plotted using the program standArich v1.0 (available at http://www.ualg.pt/ccmar/maree/software.php, F. Alberto, University of Algarve, Faro, Portugal). A correspondence analysis (CA) of *F. spiralis* and *F. vesiculosus* genotypes was performed in order to identify the main alleles contributing to the distinction between the two taxa. Two principal characteristics of a multidimensional CA plot are that the eigenvalue associated with each axis represents an estimate of the mean multilocus genetic differentiation observed on the given factorial axis and that the factorial plots of alleles and genotypes can be superimposed ('joint scaling'; Guinand, 1996). In the superimposition of alleles and genotypes, the association of an allele with a cluster of genotypes is directly proportional to the diagnostic nature of that allele to this cluster. To assume such correspondence, however, it is necessary to consider also the contribution that each allele makes to the factorial axes, because variances in the CAs are not proportional to the distance between a sample and the origin. The contribution of each allele to the distinction between *F. spiralis* and *F. vesiculosus* was thus determined as their coordinate on, and their relative contribution to, the factorial axis of the CA along which *F. spiralis* to *F. vesiculosus* individuals were differentiated. The CA on the matrix of allele counts per sample was performed using the procedure AFC in the software GENETIX 4.05 (Belkhir *et al*., 2001). Because the weight of rare alleles tends to be increased in CAs, alleles whose frequency was less than 5% in the total sample were treated as supplementary elements.

Genetic diversity at the population and species levels was estimated as mean number of alleles per locus (A), observed and expected unbiased heterozygosities (H_O and $\hat{H}$ respectively), and by the proportion of loci that were polymorphic at the 99% level. Nei's unbiased gene diversity statistics (Nei, 1978; Nei & Chesser, 1983) were

Table 1. Characteristics of five new *Fucus spiralis* microsatellite loci compared to those developed by Engel *et al*. (2003). Sequences of *Fucus spiralis* microsatellite loci have GenBank accession numbers DQ314269–DQ314273

					F. spiralis (163 inds)			*F. vesiculosus* (168 inds)		
Locus	Primer sequences (5′–3′)	Repeat array	PCR Pg Fsp/Fves[a]	[$MgCl_2$] Fsp/Fves mM[b]	*A* (Size range) (bp)	H_O	$\hat{H}$	*A* (Size range) (bp)	H_O	$\hat{H}$
Fsp1	F: TCAAAAGCCAGCAGGGGTG R: TCTTCTGGGAGCTGTAAAATAGTC	$(AG)_{11}$	(1) $x = 40$	1.5	4 (140–150)	0.04	0.46	9 (140–158)	0.55	0.82
Fsp2	F: GCATCTGGTGTCATTCCTTGTTC R: TTGTTTGAGTGCCACCTTGC	$(TC)_6CT(TC)_3G(CT)_5$	(1) $x = 35/30$	1.5	8 (155–192)	0.45	0.67	11 (153–194)	0.52	0.75
Fsp3	F: TGGAGGCCCTCCACAGCC R: TGCATTGTATGTCCTGTCCC	$(AG)_{12}$	(2) 50°C	2.0/2.5	12 (108–148)	0.11	0.74	12 (108–130)	0.30	0.87
Fsp4	F: ATGACCGGGCCGGATTGC R: GTGCTTCCCCTCCTTGTTCTGTTG	$(AG)_6AA(AG)_{22}$	(4)	2.0/2.5	9 (130–168)	0.14	0.83	14 (128–168)	0.58	0.66
Fsp5	F: GGACAGATGCGGTCGGTAGTATTGC R: GGGGAGAACCCTTTCACACACTTGG	$(TC)_4G(CT)_3TT(CT)_3T(TC)_6TT(TC)_8$	(1) $x = 40$	1.5	3 (177–201)	0.00	0.28	–	–	–
L20	Engel *et al*., 2003	$CTGG(CTG)_8(TTG)_3CTT(CTG)_2$	(2) 54°C	2.0	8 (120–165)	0.04	0.72	13 (120–168)	0.47	0.72
L58	Engel *et al*., 2003	$(GA)_{19}$	(2) 52°C	2.0	3 (107–111)	0.05	0.09	6 (105–115)	0.34	0.72
L94	Engel *et al*., 2003	$(GCA)_3GACGAT(GCA)_5$ $ACA(GCA)_5[GCT(VCA)_6]_2$	(3)	2.0	6 (154–178)	0.07	0.22	10 (154–187)	0.45	0.65
L38	Engel *et al*., 2003	$(GCT)_{11}GCC(GVT)_7$	(3)	2.0	2 (188–209)	0.02	0.02	5 (188–218)	0.34	0.67
L78	Engel *et al*., 2003	$(TGC)_{11}TGT(TGC)_2$ $TGGCGGTGCTGT(TGC)_3$	(3)	2.0	6 (138–174)	0.06	0.44	8 (147–177)	0.48	0.65
Mean over loci	All loci				6.1	0.10	0.45	9.8	0.45	0.74
Mean over loci	This study only				7.2	0.15	0.59	11.5	0.49	0.78
Mean over loci	Engel *et al*., 2003 only				5.0	0.05	0.30	8.4	0.42	0.70

[a]PCR pg Fsp/Fves: PCR programs for *F. spiralis* and *F. vesiculosus*, respectively: (1) *x* cycles of 30 s at 94°C, 40 s at 55°C, 30 s at 72°C; (2) 35 cycles of 30 s at 94°C, 30 s at T_m, 30 s at 72°C; (3) 40 cycles of 40 s at 94°C, 40 s at 55°C, 30 s at 72°C; (4) two cycles of 30 s at 94°C, 30 s at 62°C, 30 s at 72°C, 14 cycles of 0.5°C touchdown: 15 s at 94°C, 15 s at 62–55°C, 15 s at 72°C, 24 cycles of 15 s at 94°C, 15 s at 55°C, 15 s at 72°C.
[b][$MgCl_2$] Fsp/Fves mM: $MgCl_2$ concentration in PCR reactions for *F. spiralis* and *F. vesiculosus*, respectively.
H_O: observed heterozygosity. $\hat{H}$: expected unbiased heterozygosities. *A*: size range (bp): mean allele number per locus and alleles size range.

Fig. 1. Study sites of *Fucus spiralis* and *Fucus vesiculosus* across their south European distribution.

also estimated for *F. spiralis* and *F. vesiculosus* and differentiated total species-wide genetic diversity (H_T), genetic diversity within populations (H_S), and the proportion of the total diversity among populations (G_{ST}). All estimates were calculated using GENETIX 4.05 (Belkhir *et al.*, 2001). Inbreeding coefficients F_{IS} (Wright, 1969) were estimated within locations at each locus and across all loci by f (Weir & Cockerham, 1984). Inbreeding coefficients and genotypic disequilibrium coefficients were tested for departure from the null hypothesis (Hardy-Weinberg and linkage equilibria, $f=0$, $R=0$) using permutations (2,000 permutations of alleles or genotypes, within each sample) using GENETIX 4.05. The differences of mean genetic diversity and inbreeding coefficient parameters between *F. spiralis* and *F. vesiculosus* were tested using paired t-tests across loci per location and at the species level (SPSS Inc., 1989–2002). Wilcoxon rank tests were performed to compare genetic diversities and inbreeding coefficients between *F. spiralis* and *F. vesiculosus* across populations along the species latitudinal range analysed in this study. Differences of inbreeding coefficient parameters (f) between the two species per location were also tested by comparing the two means and their 95% confidence intervals obtained by 2000 bootstraps over individuals for each population (GENETIX 4.05). The correlations between latitude and genetic diversity or inbreeding coefficient were tested using regression analyses (SPSS Inc., 1989–2002).

Results

Five new polymorphic loci were identified from the *F. spiralis* library (Table 1). Each locus was checked for null alleles (van Oosterhout *et al.*, 2006) in each sampled population. Null alleles were suspected in some samples at loci Fsp1, Fsp3, L20, L78 (*F. spiralis* and *F. vesiculosus*), Fsp4, Fsp5, L94 (*F. spiralis*) and Fsp2, L58, L38 (*F. vesiculosus*) (Table 2). However, it is possible that there were no null alleles at these loci since the observation of null alleles at each locus was not consistent across locations or species, except for locus Fsp3. These results could be due, for example, to the potential underestimation of the inbreeding coefficients used in the estimation of null allele frequencies (van Oosterhout *et al.*, 2006). In contrast, PCR amplification of Fsp3 was inconsistent and null alleles in this locus were frequent in most samples of both *F. spiralis* and *F. vesiculosus*.

The resolution of the combination of markers was improved by the addition of five new microsatellite loci, in particular in *F. spiralis*. Higher heterozygosity (H_O) and gene diversity (A and $\hat{H}$) were observed using the five *F. spiralis* microsatellite loci in comparison to the previously developed loci (Engel *et al.*, 2003) in both species (at the species level, Table 1). The mean number of alleles across all loci was estimated as 6.1 and 9.8 for *F. spiralis* and *F. vesiculosus*, respectively, whereas it was estimated as 5.0 and 8.4, respectively, with the initially developed loci (Table 1). Similarly, estimates of the observed and expected unbiased heterozygosities ($H_O/\hat{H}$) increased from 0.05/0.30 and 0.42/0.70 to 0.10/0.45 and 0.45/0.74 for *F. spiralis* and *F. vesiculosus*, respectively, with the use of the new microsatellite loci (Table 1). However, none of these trends was statistically significant (one- and two-tailed t-tests, $p > 0.10$). Higher heterozygosity and gene diversity could also be observed for di-nucleotide microsatellite loci in comparison to tri-nucleotide microsatellite loci except for locus L20. However, this difference was not statistically significant (one- and two-tailed t-tests, $p > 0.10$; except for *F. vesiculosus* unbiased expected heterozygosity, significant one-tailed t-test, $p = 0.08$). The addition of the new *F. spiralis* microsatellite loci to the previous microsatellite combination increased the power of detection of new multilocus genotypes (genotypic richness from 0.14 to 0.40 [eight loci], 0.52 [10 loci] for *F. spiralis* and from 0.93 to 1 [eight and nine loci] for *F. vesiculosus*; Fig. 2). Using the new combination of loci, each individual of the dioecious species *F. vesiculosus* was described by a unique multilocus genotype. However, in the species capable of selfing, *F. spiralis*, many individuals could still not be distinguished based on their 10-microsatellite loci genotypes.

The use of Fsp5 primers in *F. vesiculosus* resulted in either no amplification (Fig. 3) or the amplification of DNA fragments of unexpected sizes. Sequencing of the fragments amplified in both species using two different primer pairs, Fsp5 primers and a new primer pair designed in the

Table 2. Inbreeding coefficient estimates (f) and estimated frequency of null alleles (*Nll*) per microsatellite locus within *Fucus spiralis* and *Fucus vesiculosus* sampling sites. Bold type indicates significant departure from 0 at $p = 0.05$. –: monomorphic locus

	Fsp1		Fsp2		Fsp3		Fsp4		Fsp5		L20		L58		L94		L38		L78	
Populations	*f*	*Nll*	*f*	*Nll*	*f*	*Nll*	*f*	*Nll*	*f*	*Nll*	*f*	*Nll*	*f*	*Nll*	*f*	*Nll*	*f*	*Nll*	*f*	*Nll*
F. spiralis																				
La Crèche	–		**0.83**		**0.60**	*0.10*	**0.73**	*0.07*	–		–		–		–		–		–	
La Guardia	0.34		**0.57**		**0.36**	*0.38*	**0.60**	*0.21*	–		**1.00**		**0.71**		**0.57**	*0.07*	0.00		**0.46**	*0.16*
Viana do Castelo	**0.58**	*0.10*	**0.65**		**0.70**		**0.53**	*0.09*	**1.00**	*0.02*	**0.81**	*0.02*	0.17		**0.57**	*0.20*	–		0.22	
Mindelo	–		**0.67**		0.00		**0.69**		**1.00**		−0.02		0.00		0.00		-0.02		0.00	
Ribeira d'Ilhas	**0.89**	*0.23*	**-0.75**		**1.00**		**0.82**	*0.26*	–		**0.88**	*0.31*	–		–		–		–	
Almograve	–		-0.01		**0.92**	*0.11*	**0.85**	*0.19*	–		**0.91**	*0.22*	–		–		–		–	
Albufeira	–		**−0.82**		**1.00**	0.36	−0.03		**1.00**		–		–		−0.01		−0.01		–	
F. vesiculosus																				
La Crèche	0.13		−0.10		**0.59**	*0.43*	**0.24**				−0.07		0.00		0.06		**0.32**	*0.18*	**0.30**	*0.12*
La Guardia	**−0.32**		0.06		**0.69**	*0.42*	0.20				**0.55**	*0.36*	−0.12		0.17		−0.12		0.05	
Viana do Castelo	0.18		**0.34**	*0.12*	**0.62**	*0.43*	**−0.34**				0.15		0.03		−0.21		0.25		−0.06	
Mindelo	**0.41**	*0.34*	−0.09		**0.56**	*0.40*	0.00				−0.01		0.66		−0.14		−0.23		−0.15	
Alcochete	−0.20		0.03		**0.50**	*0.30*	0.06				−0.01		0.36		−0.05		−0.05		0.05	
Ria Formosa	−0.16		−0.05		−0.25		−0.17				−0.18		–		−0.04		−0.19		0.14	
Tavira	**0.44**	*0.26*	0.06		1.00		−0.36				−0.02		**0.34**	*0.09*	0.22		**0.80**	*0.32*	−0.08	

flanking regions of the original *F. spiralis* Fsp5 clone sequence (GenBank accession number DQ314273), indicated that the fragment amplified in *F. vesiculosus* was not homologous to *F. spiralis* Fsp5 microsatellite locus (data not shown). We concluded that locus Fsp5 was absent in the genome of *F. vesiculosus*.

Across the other loci, allele frequencies at almost all loci differentiated the two species even though most alleles observed in *F. spiralis* could be found in *F. vesiculosus* samples (Fig. 3). The alleles that contribute the most to the distinction between *F. spiralis* and *F. vesiculosus* genotypes, as a proxy for their species specificity, were determined using their coordinate along and their relative contribution to the first axis of the CA of the total microsatellite data (alleles less frequent than 5% in the total sample treated as supplementary elements; Fig. 3). The first component of the CA, which describes the most variation of all axes, explained 17.25% of the total variation and was able to distinguish 99% of *F. spiralis* from *F. vesiculosus* genotypes (data not shown). Alleles showing high relative contributions to the first axis of the CA were observed at all loci except at locus Fsp3 (Fig. 3). The combination of microsatellite loci allowed an easy distinction between the two species.

At locus Fsp3, PCR amplification was inconsistent and high frequencies of null alleles were suspected in this locus. For this reason, and since Fsp5 did not amplify in *F. vesiculosus*, population genetic analyses were performed using the remaining eight microsatellite loci. Genetic diversities (H_O, $\hat{H}$ and A; H_S and H_T)

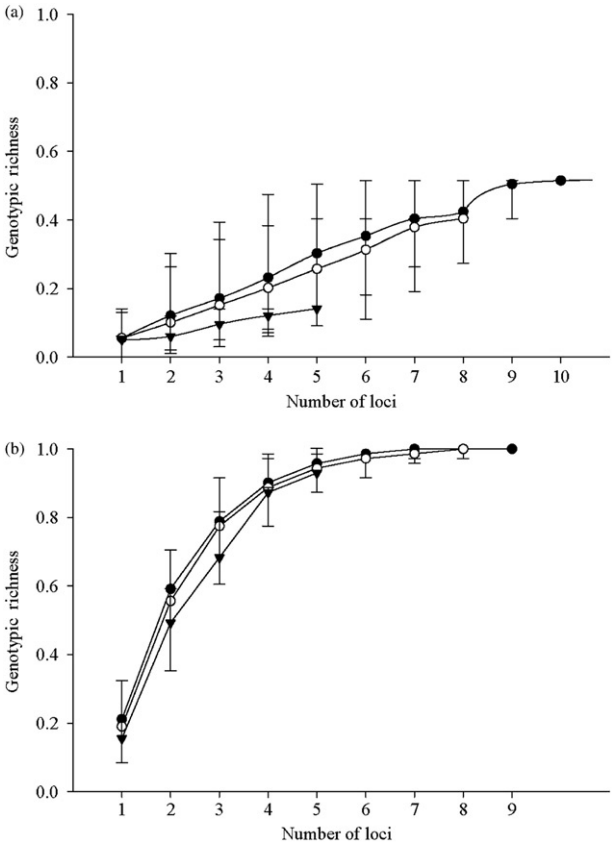

Fig. 2. Distribution of genotypic richness for all possible combinations of microsatellite loci for (a) *Fucus spiralis* and (b) *Fucus vesiculosus*, described as median genotypic richness R (error bars: maximum–minimum). Estimates used combinations of: 10 and nine microsatellite loci available in this study for *Fucus spiralis* and *Fucus vesiculosus*, respectively (●); eight microsatellite loci used in the population genetic analyses (○); or five microsatellite loci previously developed by Engel *et al.* (2003; ▼).

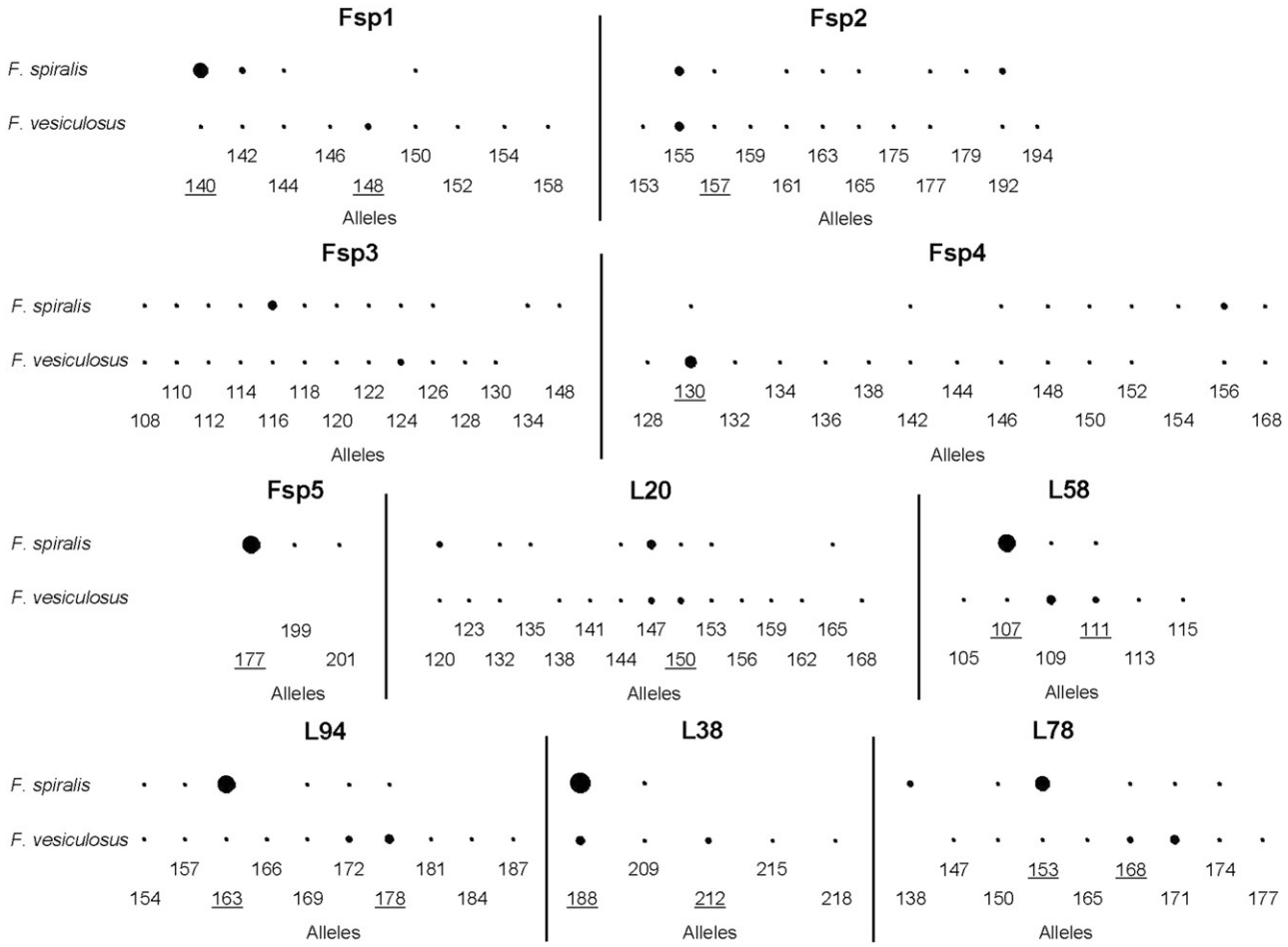

Fig. 3. Allele frequencies at five microsatellite loci developed in this study (Fsp1–5) and five microsatellite loci previously developed by Engel *et al*. (2003) (L20, L58, etc.) calculated for the total number of individuals within *Fucus spiralis* and *F. vesiculosus*. Numbers on the *x*-axis are allele sizes in bp for each locus. Each circle indicates presence of corresponding allele; diameter of circle represents frequency of that allele in the species. Alleles that contribute the most to the distinction between *F. spiralis* and *F. vesiculosus* genotypes were determined by their coordinates along the axis that differentiated *F. spiralis* from *F. vesiculosus* individuals on the correspondence analysis (CA; first axis) and their relative contribution to this axis (data not shown). Alleles underlined are those with the highest relative contribution to the first factorial axis of the CA (arbitrarily the highest 15% of the relative contribution values). Alleles characteristic of each species, defined as the most contributing alleles (arbitrarily the highest 5% of the relative contribution values to the first axis), are Fsp5 177, L58 107 and 111, and L94 163 and 178.

were significantly lower for *F. spiralis* than for *F. vesiculosus* at the species and population levels (Tables 1, 3, 4; *t*-tests and Wilcoxon sign tests across eight loci, $p < 0.05$). In contrast, the proportion of the total diversity among populations (G_{ST}) was significantly higher in the hermaphroditic than in the dioecious species (*t*-test, $p < 0.05$; Table 4). Inbreeding coefficient estimates f were high and significant in all *F. spiralis* samples (Tables 2, 3) indicating very strong departure from Hardy-Weinberg equilibrium. Some significant heterozygote deficiencies were observed in *F. vesiculosus* samples but with much lower estimates than in *F. spiralis* (Tables 2, 3). f estimates were significantly higher in *F. spiralis* populations than in *F. vesiculosus* populations (Tables 2, 3; *t*-tests, bootstraps and Wilcoxon sign tests across eight loci; $p < 0.05$), except in *F. spiralis* populations sampled at the southern limit of the species distribution, Albufeira and Ribeira d'Ilhas (Fig. 1). In these two locations, *F. spiralis* samples presented a large heterozygote excess at locus Fsp2 (Table 2). *Fucus spiralis* showed a high number of significant linkage disequilibria (LD) between pairs of loci, whereas LD were rarely observed in *F. vesiculosus* (Table 3). In *F. spiralis*, several loci were fixed in many locations (Table 2). *Fucus spiralis* did not show any differences in gene diversity and inbreeding level along the latitudinal range of the species, for the locations sampled in this study (Table 3). In contrast, *F. vesiculosus* displayed a significant trend towards lower gene diversity approaching

Table 3. Multilocus microsatellite estimators of genetic diversity, inbreeding coefficients and linkage disequilibria within *Fucus spiralis* and *Fucus vesiculosus* sampling sites using eight loci (L20, L58, L94, L38, L78, Fsp1, 2 & 4)

Populations		*n*	*A*	*P* (0.99)	H_O	$\hat{H}$	*f* (95% CI)	LD/28 (fixed)
F. spiralis (eight loci)								
North France	La Crèche	24	1.5	0.3	0.03	0.12	**0.78** (0.51–0.96)	(27)
North-west Spain	La Guardia	21	3.0	1.0	0.12	0.28	**0.57** (0.34–0.78)	13 (2)
North Portugal	Viana do Castelo	22	3.4	0.9	0.16	0.33	**0.54** (0.23–0.78)	13 (7)
	Mindelo	24	2.3	0.9	0.08	0.16	**0.53** (0.27–0.82)	4 (8)
Central Portugal	Ribeira d'Ilhas	24	1.6	0.5	0.14	0.23	**0.40** (0.16–0.51)	1 (22)
	Almograve	24	1.8	0.4	0.10	0.22	**0.52** (0.32–0.65)	2 (25)
South Portugal	Albufeira	24	1.8	0.4	0.15	0.10	**−0.58** (−0.81–0.42)	(25)
F. spiralis at the species level		163	5.8	0.88	0.11	0.43		
F. vesiculosus (eight loci)								
North France	La Crèche	24	7.0	1.0	0.67	0.75	**0.10** (0.01–0.15)	2
North-west Spain	La Guardia	24	4.8	1.0	0.44	0.48)	**0.10** (−0.07–0.22)	1
North Portugal	Viana do Castelo	24	4.3	1.0	0.56	0.59	0.05	2
	Mindelo	24	3.8	1.0	0.42	0.42	0.01	2
Central Portugal	Alcochete	24	3.1	1.0	0.39	0.40	0.03	2
South Portugal	Ria Formosa	24	2.9	0.9	0.45	0.41	−0.09	9 (7)
	Tavira	24	2.8	1.0	0.35	0.45	**0.21** (0.08–0.30)	2
F. vesiculosus at the species level		168	9.5	1.0	0.47	0.72		

n: sample size. *A*: mean number of alleles per locus. *P* (0.99): 99% polymorphism. H_O and $\hat{H}$: observed and expected unbiased heterozygosities, respectively. *f* is the F_{IS} estimate and (95% CI) its 95% confidence interval estimated using 2,000 bootstraps over individuals, calculated only for significant departures from 0. LD is the number of significant correlations between pairs of loci (linkage disequilibria) and in brackets are the pair-wise comparisons where one locus is fixed. Bold type indicates significant departure from 0 at $p = 0.05$.

Table 4. Gene diversity statistics based on the estimators of Nei (1978; Nei & Chesser, 1983) for *Fucus spiralis* and *Fucus vesiculosus* populations. H_S: genetic diversity within populations, H_T: total genetic diversity and G_{ST}: proportion of the total diversity among populations

Species (eight loci)	H_S	H_T	G_{ST}
F. spiralis	0.21	0.44	0.52
F. vesiculosus	0.50	0.72	0.30

its southern limit of distribution (Table 3; regression coefficients for *A*: $r^2 = 0.81$, $p < 0.05$; H_O: $r^2 = 0.53$, $p < 0.05$; and $\hat{H}$: $r^2 = 0.46$, $p = 0.06$).

Discussion

Marker resolution for distinguishing genotypes

The use of new loci specifically designed for the hermaphroditic species *F. spiralis* increased the estimates of both genetic and genotypic diversities in both *F. spiralis* and *F. vesiculosus*. In the dioecious *F. vesiculosus*, the new markers allowed the detection of every individual as a unique multilocus genotype whereas, for *F. spiralis*, many individuals still shared the same multilocus genotypes. Despite this, identical genotypes in *F. spiralis* must originate from different sexual reproductive events as sexual reproduction is the only means of propagation known to occur in this species.

Potential diagnostic marker for F. spiralis *and* F. vesiculosus

Fsp5 appears to be a promising diagnostic marker to differentiate these two closely related species and would be the first diagnostic marker observed so far for these species across their distributional range. Species-specific allele frequencies were also identified at all loci except Fsp3 and the combination of microsatellite loci allowed the distinction between the two species across their range. This is consistent with the species-specific alleles already identified by Billard *et al.*, (2005*b*) to distinguish *F. spiralis*, *F. vesiculosus* and *F. ceranoides* in Brittany (France).

Marker resolution for mating system

Previous population genetic analyses of *F. spiralis* and *F. vesiculosus* suggested some selfing or inbreeding in populations in Maine, USA (Coleman & Brawley, 2005) and very high rates of self-fertilization in North Portugal and North France (Engel *et al.*, 2005) in *F. spiralis*. However, because of the low polymorphism or monomorphism of the loci analysed within populations, estimates of inbreeding coefficients were not always possible. In the present study, marker variability remained low and estimates of genetic inbreeding coefficients were high in hermaphroditic *F. spiralis* compared with dioecious *F. vesiculosus* even with the use of *F. spiralis*-specific microsatellite loci. It is unlikely, therefore,

that the low polymorphism or monomorphism observed by Engel *et al.* (2005) and Coleman & Brawley (2005) were the consequence of the cross-amplification of the markers in *F. spiralis*. Rather, the lower levels of polymorphism of those microsatellites (Engel *et al.*, 2003) are probably due to the mutation rate of their repeat motif (mostly tri-nucleotides) and the reproductive mode of the species. Mutation rates of dinucleotide microsatellites, such as the *F. spiralis* loci described here, are expected to be higher than those of tri-nucleotide microsatellites (Chakraborty *et al.*, 1997; Ellegren, 2000), although the variability of di- compared to trinucleotide markers in our study was not significantly higher. Importantly, the high variance of the inbreeding coefficient estimates depending on the locus or combination of loci analysed, as shown in this study, highlights one of the pitfalls of estimating selfing rates using indirect genetic measures.

F. spiralis *and* F. vesiculosus *mating systems*

The populations of the hermaphroditic *F. spiralis* showed high departures from Hardy-Weinberg equilibrium (heterozygote deficiency) independently of the marker or combination of markers analysed, and showed linkage disequilibria (LD). These are characteristic of selfing species (Hamrick & Godt, 1997; Holsinger, 2000; Charlesworth & Wright, 2001; Trouvé *et al.*, 2003) and differentiate *F. spiralis* from dioecious *Fucus* species such as *F. vesiculosus* (Wallace *et al.*, 2004; Engel *et al.*, 2005; this study) and *F. serratus* (Coyer *et al.*, 2003) for which much lower or null heterozygote deficiencies and LD were found. Because the breeding strategy is the main difference between the two sister species *F. spiralis* and *F. vesiculosus*, these results are evidence for high rates of selfing in hermaphroditic *F. spiralis*, both in marginal populations at the southern limit of distribution and in core populations. Furthermore, *F. spiralis* seems consistently to reproduce mostly by selfing throughout its distribution, as genotypic changes caused by inbreeding affect all loci in the genome, but such changes would be ephemeral if inbreeding were not continuous. Indeed, whereas inbreeding over past generations can be detected by linkage disequilibria between pairs of loci, heterozygote deficiency can be eliminated in one generation of random mating, even with extremely high frequency of homozygotes. Although *F. spiralis* reproduces mostly by selfing, however, it occasionally outcrosses, as suggested by inbreeding coefficients of less than 0.78, allowing recombination to occur. This suggests some flexibility in its reproductive strategy.

Increasing mating probability may explain a selective advantage of autogamy in *F. spiralis* across its distribution. External fertilization has a low probability of success on exposed shores and outcrossing *Fucus* species can reproduce only when/if sheltered windows of opportunity occur (Serrão *et al.*, 1996 and references therein). In addition, *F. spiralis* produces fewer sperm per egg (an order of magnitude lower) than dioecious *Fucus* species (Vernet & Harper, 1980; Billard *et al.*, 2005*a*), even further decreasing the probability of successful outcross matings on wave exposed shores. *Fucus spiralis* is also typically found higher in the intertidal than *F. vesiculosus*, and is thus exposed to more stressful environmental conditions. Selfing in *F. spiralis*, in conjunction with selection, may thus enable this species to organize specialized multilocus systems to survive in their extreme environment (Stebbins, 1950; Hawkins & Hartnoll, 1985; Takebayashi & Morrell, 2001). Indeed, desiccation tolerance was suggested to be a factor in the intertidal zonation of *Fucus* species (Dring & Brown, 1982, reviewed by Chapman 1995; Davison & Pearson, 1996).

In this study, we did not intend to estimate the actual rate of selfing in *F. spiralis*. Measures of inbreeding coefficient f (Wright, 1969; Weir & Cockerham, 1984) can provide estimates of the mating system (outcrossing rate $t=(1-f)/(1+f)$; Nei & Syakudo, 1958) based on the assumption that the population is at inbreeding equilibrium and that selfing is the only form of inbreeding occurring (Ritland, 1983). However, many factors other than mating system may contribute to inbreeding and genetic estimation of inbreeding from molecular marker analyses should only be used as a preliminary means of studying a species mating system (Ritland, 1983). Significant heterozygote deficiencies in fucoid algae are likely explained by biparental inbreeding due to short gamete and zygote dispersal, potential reproductive synchrony of related individuals or Wahlund effect (Chapman, 1995; Pearson & Brawley, 1996; Serrão *et al.*, 1997; Arrontes, 2002; Coyer *et al.*, 2003; Wallace *et al.*, 2004; Engel *et al.*, 2005). Indeed, fine-scale spatial structure, consistent with low dispersal potential, was observed in populations of *F. spiralis* (Engel *et al.*, 2005) in contrast with *F. vesiculosus* in which dispersal was not limited at the spatial scales analysed (Engel *et al.*, 2005; see also Coyer *et al.*, 2003 for the same observation in *F. serratus*). Variability in reproductive success, dispersal, settlement and recruitment or temporally varying selection in each generation, may also create inbreeding and has been proposed for dioecious *Fucus* species (Coyer *et al.*, 2003; Engel *et al.*, 2005). Finally, hybridization and introgression between *F. spiralis*

and *F. vesiculosus* (Engel *et al*., 2005; Billard *et al*., 2005*a*; Coyer *et al*., 2006*a*, *b*) may contribute to heterozygote deficiencies (Engel *et al*., 2005).

Heterozygote excesses in populations of F. spiralis *at its limit of distribution*

In Ribeira d'Ilhas and Albufeira (Portugal), high heterozygote excesses were observed for *F. spiralis* at locus Fsp2, whereas other samples at this locus or other loci showed heterozygote deficiencies. A few hypotheses could be suggested: (i) associative overdominance favouring heterozygotes can explain heterozygote excess (Nei, 1987); however, this would require locus Fsp2 to be linked to a selected gene, and selection acting on this gene would have to be very strong to generate such excess of heterozygote genotypes. (ii) Heterozygote excesses are also reported in asexual populations of clonal organisms due to high-frequency heterozygote clones. The potential for asexual reproduction has never been shown in *F. spiralis*, but it is not impossible since it has been revealed in another *Fucus* species (Tatarenkov *et al*., 2005). However, it depends upon reattachment of vegetative fragments, and is thus highly unlikely on exposed high intertidal shores. (iii) We may have amplified several copies of the same locus (polyploidy, such as in *Fucus* polyploid ecads from marginal populations in Ireland, Coyer *et al*., 2006*b*). However, no evidence of polyploidy has been found at other loci or in other populations in our study. (iv) Finally, the primer pair may have amplified two distinct loci, although there is no evidence for this in other populations sharing the same alleles, or in *F. vesiculosus*. None of these hypotheses is satisfactory, and the lack of a firm theoretical basis for heterozygote excess in natural populations makes these observations difficult to explain.

Implications of mating system for genetic diversity at population and species levels

Mating systems and spatial structure affect genetic diversity within and between populations. Patterns of reduced genetic diversity at the population and the species levels and high genetic structure are expected in selfing compared to outcrossing species (Charlesworth & Charlesworth, 1995; Awadalla & Ritland, 1997; Hamrick & Godt, 1997; Charlesworth & Pannell, 2001; Charlesworth & Wright, 2001; Sweigart & Willis, 2003). Expected genetic diversity patterns were confirmed in the *Fucus* species complex studied here. For the hermaphroditic species *F. spiralis*, measures of genetic diversity within sampled populations were about half the estimates observed in its outcrossing sister species *F. vesiculosus*, independently of the locus or the location analysed. The very low genetic diversity in *F. spiralis* might indicate a lower ability to adapt to environmental changes (Stebbins, 1957; Charlesworth & Charlesworth, 1995; Holsinger, 2000; Takebayashi & Morrell, 2001) or, alternatively, increased homozygosity that decreases the probability of fixation of the slightly deleterious alleles by increasing the effective selection coefficient, thus reducing inbreeding depression (Jarne & Charlesworth, 1993; Charlesworth & Charlesworh, 1995; Charlesworth & Wright, 2001; Takebayashi & Morrell, 2001).

At the species level, however, population isolation in inbred species may allow high diversity (Hamrick & Godt, 1997; Charlesworth & Pannell, 2001; Charlesworth & Wright, 2001), resulting in less difference in diversity between selfing and outcrossing species. This was the case in our study where total genetic diversity in *F. spiralis* was slightly higher than at local scales, although it was still significantly lower than the total diversity observed in *F. vesiculosus* across the same geographic range. Among populations, on the other hand, the proportion of the total diversity observed was significantly higher in *F. spiralis*. Frequent population isolation in selfing species, bottlenecks or recent colonization events, coupled with selfing, reduce effective population sizes, but increase the effect of genetic drift within populations and thus the level of genetic structure among populations at the species level (Slatkin, 1985; Hamrick & Godt, 1997).

In addition to the mating system, asymmetrical introgression of the *F. spiralis* genome in the non-selfing *F. vesiculosus* genome (Engel *et al*., 2005) may increase genetic diversity in populations of *F. vesiculosus* (Sweigart & Willis, 2003; Engel *et al*., 2005). At a broad geographical scale for this species, and in contrast with *F. spiralis*, spatial structure has affected genetic diversity. Peripheral populations of *F. vesiculosus* at the southern limit of distribution retain lower genetic diversity compared to core populations. These marginal populations are allopatric with *F. spiralis* thus precluding introgression. However, the low diversity of marginal populations is likely to result from stronger genetic drift affecting small isolated populations in the fragmented estuarine habitats occupied by *F. vesiculosus* at its southern latitudinal range (Daguin *et al*., unpublished data). Besides, founder effects may have influenced genetic diversity, as small populations at their ecological limit have a high probability of extinction events.

Conclusions

The newly developed *F. spiralis* microsatellite markers presented in this study allowed us to increase the resolution of our combination of markers for the study of *F. spiralis* and *F. vesiculosus* populations. Our study is consistent with previous results and shows that *F. spiralis* reproduces mostly through self-fertilization along European shores from northern France to the southern Iberian Peninsula. *Fucus spiralis* shows very low levels of within-population genetic diversity along the latitudinal range sampled, and high genetic structure among populations at the species level compared with its dioecious sister species *F. vesiculosus*. In contrast, the outcrossing species *F. vesiculosus* retains higher levels of genetic diversity that decline towards the southern latitudinal edge of the species distribution.

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References

Arnaud-Haond, S., Alberto, F., Teixeira, S., Procaccini, G., Serrao, E.A. & Duarte, C.M. (2005). Assessing genetic diversity in clonal organisms: low diversity or low resolution? Combining power and cost efficiency in selecting markers. *J. Hered.*, **96**: 434–440.

Arrontes, J. (2002). Mechanisms of range expansion in the intertidal brown alga *Fucus serratus* in northern Spain. *Mar. Biol.*, **141**: 1059–1067.

Awadalla, P. & Ritland, K. (1997). Microsatellite variation and evolution in the *Mimulus guttatus* species complex with contrasting mating systems. *Mol. Biol. Evol.*, **14**: 1023–1034.

Belkhir, K., Borsa, P., Goudet, J., Chikhi, L. & Bonhomme, F. (2001). GENETIX, logiciel sous Windows^{TM} pour la genetique des populations, *Laboratoire Genome et Populations*. Universite de Montpellier II, France.

Berndt, M.L., Callow, J.A. & Brawley, S.H. (2002). Gamete concentrations and timing and success of fertilization in a rocky shore seaweed. *Mar. Ecol. Prog. Ser.*, **226**: 273–285.

Billard, E., Daguin, C., Pearson, G., Serrao, E., Engel, C. & Valero, M. (2005*a*). Genetic isolation between three closely related taxa: *Fucus vesiculosus*, *F. spiralis*, and *F. ceranoides* (Phaeophyceae). *J. Phycol.*, **41**: 900–905.

Billard, E., Serrão, E.A., Pearson, G.A., Engel, C.R., Destombe, C. & Valero, M. (2005*b*). Analysis of sexual phenotype and prezygotic fertility in natural populations of *Fucus spiralis*, *F. vesiculosus*, (Fucaceae, Phaeophyceae) and their putative hybrids. *Eur. J. Phycol.*, **40**: 397–407.

Burrows, E.M. & Logde, S.M. (1951). Autecology and the species problem in *Fucus*. *J. Mar. Biol. Assoc. UK*, **30**: 161–175.

Chakraborty, R., Kimmel, M., Stivers, D.N., Davison, L.J. & Deka, R. (1997). Relative mutation rates at di-, tri-, and tetranucleotide microsatellite loci. *Proc. Natl. Acad. Sci. USA*, **94**: 1041–1046.

Chapman, A.R.O. (1995). Functional ecology of fucoid algae: twenty three years of progress. *Phycologia*, **34**: 1–32.

Charlesworth, B. (1980). The cost of sex in relation to mating system. *J. Theor. Biol.*, **84**: 655–671.

Charlesworth, B. (1992). Evolutionary rates in partially self fertilizing species. *Am. Nat.*, **140**: 126–148.

Charlesworth, D. & Charlesworth, B. (1995). Quantitative genetics in plants: the effect of the breeding system on genetic variability. *Evolution*, **49**: 911–920.

Charlesworth, D. & Pannell, J.R. (2001). Mating system and population genetic structure in the light of coalescent theory. In *Integrating Ecology and Evolution in a Spatial Context. British Ecological Society Special Symposium 2000* (Silvertown, J. and Antonovics, J., editors), 73–95. Blackwell Science Ltd, Oxford, UK.

Charlesworth, D. & Wright, S.I. (2001). Breeding systems and genome evolution. *Curr. Opin. Genet. Dev.*, **11**: 685–690.

Coleman, M.A. & Brawley, S.H. (2005). Are life history characteristics good predictors of genetic diversity and structure? A case study of the intertidal alga *Fucus spiralis* (Heterokontophyta; Phaeophyceae). *J. Phycol.*, **41**: 753–762.

Coyer, J.A., Veldsink, J.H., Jones, K., Stam, W.T. & Olsen, J.L. (2002). Characterization of microsatellite loci in the marine seaweeds, *Fucus serratus* and *F. evanescens* (Heterokontophyta; Fucaceae). *Mol. Ecol. Notes*, **2**: 35–37.

Coyer, J.A., Peters, A.F., Stam, W.T. & Olsen, J.L. (2003). Post-ice age recolonization and differentiation of *Fucus serratus* L. (Phaeophyceae; Fucaceae) populations in Northern Europe. *Mol. Ecol.*, **12**: 1817–1829.

Coyer, J.A., Hoarau, G., Oudot-Le Secq, M.-P., Stam, W.T. & Olsen, J.L. (2006*a*). A mtDNA-based phylogeny of the brown algal genus *Fucus* (Heterokontophyta; Phaeophyta). *Mol. Phyl. Evol.*, **39**: 209–222.

Coyer, J.A., Hoarau, G., Pearson, G.A., Serrão, E.A., Stam, W.T. & Olsen, J.L. (2006*b*). Convergent adaptation to a marginal habitat by homoploid hybrids and polyploidy ecads in the seaweed genus, *Fucus*. *Biol. Lett.*, **2**: 405–408.

Davison, I.R. & Pearson, G.A. (1996). Stress tolerance in intertidal seaweeds. *J. Phycol.*, **32**: 197–211.

Dorken, M.E. & Eckert, C.G. (2001). Severely reduced sexual reproduction in northern populations of a clonal plant, *Decodon verticillatus* (Lythraceae). *J. Ecol.*, **89**: 339–350.

Doyle, J.J. & Doyle, J.L. (1987). A rapid DNA isolation procedure from small quantities of fresh leaf tissues. *Phytochem. Bull.*, **19**: 11–15.

Dring, M.J. & Brown, F.A. (1982). Photosynthesis of intertidal brown algae during and after periods of emersion: a renewed search for physiological causes of zonation. *Mar. Ecol. Prog. Ser.*, **8**: 301–308.

Ellegren, H. (2000). Microsatellite mutations in the germline: implications for evolutionary inference. *Trends Genet.*, **16**: 551–558.

Engel, C.R., Brawley, S.H., Edwards, K.J. & Serrao, E. (2003). Isolation and cross-species amplification of microsatellite loci from the fucoid seaweeds *Fucus vesiculosus*, *F. serratus* and *Ascophyllum nodosum* (Heterokontophyta, Fucaceae). *Mol. Ecol. Notes*, **3**: 180–182.

Engel, C.R., Daguin, C. & Serrao, E.A. (2005). Genetic entities and mating system in hermaphroditic *Fucus spiralis* and its close dioecious relative *F. vesiculosus* (Fucaceae, Phaeophyceae). *Mol. Ecol.*, **14**: 2033–2046.

Engel, C.R., Daguin, C. & Serrao, E.A. (2006). When is a hybrid a hybrid? A counter-reply to Wallace *et al. Mol. Ecol.*, **15**: 3481–3482.

Guinand, B. (1996). Use of a multivariate model using allele frequency distributions to analyse patterns of genetic differentiation among populations. *Biol. J. Linnean Soc.*, **58**: 173–195.

Hamrick, J.L. & Godt, M.J.W. (1997). Effects of life history traits on genetic diversity in plant species. In *Plant Life Histories: Ecology, Phylogeny, and Evolution* (Silvertown, J.W., Harper, J.L., and Franco, M., editors), 112–118. Cambridge University Press, Cambridge, UK.

Hawkins, S.J. & Hartnoll, R.G. (1985). Factors determining the upper limits of intertidal canopy forming algae. *Mar. Ecol. Prog. Ser.*, **20**: 265–271.

Holsinger, K.E. (2000). Reproductive systems and evolution in vascular plants. *Proc. Natl. Acad. Sci. USA*, **97**: 7037–7042.

Jarne, P. & Charlesworth, D. (1993). The evolution of the selfing rate in functionally hermaphrodite plants and animals. *Annu. Rev. Ecol. Syst.*, **24**: 441–466.

Kniep, H. (1925). Uber *Fucus* Bastarde. *Flora*, **118**: 331–338.

Ladah, L., Bermudez, R., Pearson, G. & Serrao, E. (2003). Fertilization success and recruitment of dioecious and hermaphroditic fucoid seaweeds with contrasting distributions near their southern limit. *Mar. Ecol. Prog. Ser.*, **262**: 173–183.

Maynard Smith, J. (1978). *The Evolution of Sex*. Cambridge University Press, Cambridge, UK.

Müller, D.G. & Gassmann, G. (1985). Sexual reproduction and the role of sperm attractants in monoecious species of the brown algae Order Fucales (*Fucus*, *Hesperophycus*, *Pelvetia*, and *Pelvetiopsis*). *J. Plant Physiol.*, **118**: 401–408.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**: 583–590.

Nei, M. (1987). *Molecular Evolutionary Genetics*. Columbia University Press, New York, USA.

Nei, M. & Syakudo, K. (1958). The estimation of outcrossing in natural populations. *Jpn J. Genet.*, **33**: 46–51.

Nei, M. & Chesser, R.K. (1983). Estimation of fixation indexes and gene diversities. *Ann. Hum. Genet.*, **47**: 253–259.

Pannell, J.R. & Barrett, S.C.H. (1998). Baker's law revisited: reproductive assurance in a metapopulation. *Evolution*, **52**: 657–668.

Pannell, J.R. & Barrett, S.C.H. (2001). Effects of population size and metapopulation dynamics on a mating system polymorphism. *Theor. Popul. Biol.*, **59**: 145–155.

Pearson, G.A. & Brawley, S.H. (1996). Reproductive ecology of *Fucus distichus* (Phaeophyceae): an intertidal alga with successful external fertilization. *Mar. Ecol. Prog. Ser.*, **143**: 211–223.

Rico, C., Rico, I. & Hewitt, G. (1996). 470 million years of conservation of microsatellite loci among fish species. *Proc. R. Soc. Lond. B Biol. Sci.*, **263**: 549–557.

Ritland, K. (1983). Estimation of mating systems. In *Isozymes in Plant Genetics and Breeding* (Trankley, S.D. and Orton, T.J., editors), 289–302. Elsevier Scientific Publications, Amsterdam, The Netherlands.

Scott, G.W. & Hardy, F.G. (1994). Observations of the occurrence of hybrids between two sympatric species of fucoid algae. *Cryptogam. Algol.*, **15**: 297–305.

Serrão, E.A., Pearson, G., Kautsky, L. & Brawley, S.H. (1996). Successful external fertilization in turbulent environments. *Proc. Natl. Acad. Sci. USA*, **93**: 5286–5290.

Serrão, E.A., Kautsky, L., Lifvergren, T. & Brawley, S.H. (1997). Gamete dispersal and pre-recruitment mortality in Baltic *Fucus vesiculosus*. *Phycologia*, **36**: 101–102 [Abstract].

Serrão, E.A., Alice, L.A. & Brawley, S.H. (1999). Evolution of the Fucaceae (Phaeophyceae) inferred from nrDNA-ITS. *J. Phycol.*, **35**: 382–394.

Slatkin, M. (1985). Gene flow in natural populations. *Annu. Rev. Ecol. Syst.*, **16**: 393–430.

SPSS Inc. (1989–2002). *SPSS for Windows*, Release 11.0.1, 2001. SPSS Inc., Chicago, USA.

Stebbins, G.L. (1950). *Variation and Evolution in Plants*. Oxford University Press, London, UK.

Stebbins, G.L. (1957). Self fertilization and population variability in the higher plants. *Am. Nat.*, **861**: 337–354.

Steinkellner, H., Flush, S., Turetschek, E., Lexer, C., Streiff, R., Kremer, A., Burg, K. & Glossl, J. (1997). Identification and characterization of $(GA/CT)_n$ microsatellite loci from, *Quercus petraea*. *Plant. Mol. Biol.*, **33**: 1093–1096.

Sweigart, A.L. & Willis, J.H. (2003). Patterns of nucleotide diversity in two species of *Mimulus* are affected by mating system and asymmetric introgression. *Evolution*, **57**: 2490–2506.

Takebayashi, N. & Morrell, P.L. (2001). Is self-fertilization an evolutionary dead end? Revisiting an old hypothesis with genetic theories and a macroevolutionary approach. *Am. J. Bot.*, **88**: 1143–1150.

Tatarenkov, A., Bergstrom, L., Jonsson, R.B., Serrão, E.A., Kautsky, L. & Johannesson, K. (2005). Intriguing asexual life in marginal populations of the brown seaweed *Fucus vesiculosus*. *Mol. Ecol.*, **14**: 647–651.

Trouvé, S., Degen, L., Renaud, F. & Goudet, J. (2003). Evolutionary implications of a high selfing rate in the freshwater snail *Lymnaea truncatula*. *Evolution*, **57**: 2303–2314.

van Oosterhout, C., Hutchinson, B., Wills, D. & Shipley, P. (2003). Micro-Checker: Microsatellite Data Checking Software, *Molecular Ecology and Fisheries Genetics Laboratory*. Hull University, UK.

van Oosterhout, C., Weetman, D. & Hutchinson, W.F. (2006). Estimation and adjustment of microsatellite null alleles in nonequilibrium populations. *Mol. Ecol. Notes*, **6**: 255–256.

Vernet, P. & Harper, J.L. (1980). The costs of sex in seaweeds. *Biol. J. Linnean Soc.*, **13**: 129–138.

Wallace, A.L., Klein, A.S. & Mathieson, A.C. (2004). Determining the affinities of salt marsh fucoids using microsatellite markers: evidence of hybridization and introgression between two species of *Fucus* (Phaeophyta) in a Maine estuary. *J. Phycol.*, **40**: 1013–1027.

Wallace, A.L., Klein, A.S. & Mathieson, A.C. (2006). A reply to Engel *et al. Mol. Ecol.*, **15**: 1185–1187.

Weir, B.S. & Cockerham, C.C. (1984). Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38**: 1358–1370.

Wright, S. (1969). *Evolution and the Genetics of Populations. The Theory of Gene Frequencies*. University of Chicago Press, Chicago, USA.