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Intergametophytic selfing and microgeographic genetic structure shape populations of the intertidal red seaweed *Chondrus crispus*

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Abstract

Understanding how abiotic factors influence the spatial distribution of genetic variation provides insight into microevolutionary processes. The intertidal seascape is characterized by highly heterogeneous habitats which probably influence the partitioning of genetic variation at very small scales. The effects of tidal height on genetic variation in both the haploid (gametophytes) and diploid (tetrasporophytes) stages of the red alga *Chondrus crispus* were studied. Fronds were sampled every 25 cm within a 5 m × 5 m grid and along a 90-m transect at two shore heights (high and low) in one intertidal site in France. The multilocus genotype of 799 fronds was determined ($N_{haploid}$ = 586; $N_{diploid}$ = 213) using eight microsatellite loci to test the following hypotheses: (i) high and low shore fronds belong to genetically differentiated populations, (ii) gene flow is restricted within the high shore habitat due to tidal-influenced isolation and (iii) significant F_{IS} values are driven by life history characteristics. Pairwise F_{ST} estimates between high and low shore levels supported the hypothesis that high and low shore fronds were genetically differentiated. The high shore was characterized by the occurrence of within-shore genetic differentiation, reduced genetic diversity and increased levels of intergametophytic selfing, suggesting it is a marginal environment. These results suggest at fine scales within the intertidal seascape the same mechanisms as those over the species' distributional range are at work with core and marginal population dynamics.

Keywords: algae, haploid–diploid life cycles, intergametophytic selfing, intertidal zone, mating system, population genetics, seascape influence

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Introduction

Understanding how abiotic factors influence the spatial distribution of genetic variation is one of the primary goals of molecular ecology and can provide insight into microevolutionary patterns and processes. Features, such as altitudinal clines, can affect the suitability of habitats which in turn influences gene flow, population connectivity and genetic differentiation among populations (Manel *et al.* 2003). The literature assessing spatial patterns and landscape boundaries has led to the emergence of the field of landscape genetics, where the use of multilocus genetic data is becoming more common with technical and statistical advances (reviewed in Storfer *et al.* 2010). In particular, the use of highly variable markers, such as microsatellites, accompanied by dense sampling regimes across small, topographically diverse regions, has enabled studies on the localized effects of landscapes on genetic diversity and gene flow

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(e.g. Giordano *et al.* 2007; Clarke *et al.* 2008; Arnaud *et al.* 2011).

Analogously, in the marine environment, *seascape genetic* studies have explored the correspondence between population connectivity and sea surface temperature, turbidity and salinity (e.g. Selkoe *et al.* 2008; Mendez *et al.* 2010), habitat discontinuities (e.g. Fraser *et al.* 2010), ocean current transport (e.g. Selkoe *et al.* 2010) and bathymetry (e.g. Schultz *et al.* 2008) at basin-wide (e.g. Galindo *et al.* 2006) and regional spatial scales (e.g. Selkoe *et al.* 2010). Among sessile marine organisms, genetic connectivity is influenced by a range of factors, including environmental features, such as prominent headlands, and reproductive traits, such as the mating system (Palumbi *et al.* 1997). Near-shore propagule transport has been shown to be affected by coastal topography leading to stochastic recruitment, even along linear portions of the coastline (Siegel *et al.* 2008). Contrary to the expectation of a correlation between an extended planktonic period and a high rate of larval exchange among populations, larval retention near the parental population is more frequent than suspected and may be due to hydrodynamic features (e.g. *Crepidula fornicata*, Dupont *et al.* 2007).

Many seascape genetic studies have focused on animals with planktonic stages (e.g. Galindo *et al.* 2006) or vagility (e.g. Amaral *et al.* 2012). In benthic seaweeds, the duration of spore and gamete viability is one of the major determinants of dispersal range (Gaylord *et al.* 2004, 2006). The emerging body of the literature on marine algal seascape genetics has focused on kelps (Phaeophyceae, Laminariales) and fucoids (Phaophyceae, Fucales) which are dominant, habitat-forming species in intertidal and subtidal environments. Many kelps and fucoids are capable of rafting over large distances as fertile adults, reducing genetic differentiation between populations (e.g. kelps: *Laminaria digitata*, Billot *et al.* 2003; *Macrocystis pyrifera*, Hernández-Carmona *et al.* 2006; *Durvillaea antarctica*, Fraser *et al.* 2009; or fucoids: *Fucus distichus*, Coleman & Brawley 2005; *Phyllospora comosa*, Coleman & Kelaher 2009; *Ecklonia radiata*, Coleman *et al.* 2011a). Yet, small-scale processes were found to be equally important in structuring populations. Significant population differentiation was detected among neighbouring kelp populations on rocky reefs separated by 10 s of kilometres (*L. digitata*, Billot *et al.* 2003; *M. pyrifera*, Alberto *et al.* 2010; *E. radiata*, Coleman *et al.* 2011a). Valero *et al.* (2011) concluded from a review on population structure in 17 kelp species that population connectivity was mainly dependent on their dispersal abilities and habitat characteristics (intertidal vs. subtidal), but little on their lifespan. In kelps, sperm dispersal occurs over a scale of centimetres in which pheromones released from the egg are effective (Reed 1990), whereas zoospore (leading to gametophytes) dispersal can occur over a scale of kilometres (Gaylord *et al.* 2006). Location-specific factors, such as forest density and/or habitat depth, have been shown to influence small-scale differentiation and mating system (i.e. random mating vs. inbreeding, Coleman *et al.* 2011a,b). Thus, *both* small-scale biological/ecological and large-scale oceanographic processes are important factors in understanding seaweed community dynamics.

Few genetic studies have investigated very fine-scale patterns, such as those which might occur within an intertidal rocky shore. The dynamic and heterogeneous environment of the intertidal zone probably promotes strong genetic structure due to the dramatic differences in ecophysiological and biotic features which occur over very small spatial scales (Connell 1961; Helmuth *et al.* 2011). The duration and frequency of emersion time increases dramatically with tidal height. Temperature, in particular, has been demonstrated to be one of the most important abiotic determinants of intertidal zonation (Bertness 1981; Menge & Olson 1990; Schmidt *et al.* 2008; Pearson *et al.* 2009). Organisms located higher on the shore experience longer periods of isolation at low tide accompanied by large, daily fluctuations in abiotic parameters and stress (Davison & Pearson 1996). Depending on zonational height, the emersion duration can be as long as or longer than the submersion time. By comparison, populations lower on the shore are more often immersed, relatively more buffered and, generally, characterized as more open. These differences can lead to habitat divergent selection maintaining different genetic taxa, such as has been demonstrated in the marine gastropod *Littorina saxatilis* (Johannesson 2009) or in the *Fucus spiralis/vesiculosus* species complex (Billard *et al.* 2010; Zardi *et al.* 2011). The shift in the mating system towards hermaphroditism and selfing in *Fucus* spp. inhabiting the marginal high shore was suggested to be the result of selection for increased reproductive assurance and the maintenance of locally adaptive traits (Billard *et al.* 2010; Canovas *et al.* 2011).

Density decreases for many species at upper distribution limits leading to differences in reproductive strategies (e.g. the occurrence of geographic parthenogenesis in animals, Lynch 1984; plants, Bierzychudek 1985 or a brown seaweed, Tatarenkov *et al.* 2005; a shift towards asexual reproduction in clonal plants, Eckert 2002; a shift towards selfing in hermaphroditic plants, Levin 2012; and in haploid–diploid ferns, de Groot *et al.* 2012). These patterns have been found at latitudinal scales, but also over much smaller scales, as for example, within intertidal populations of the red seaweed *Mastocarpus papillatus*. High shore females have been found to be obligately apomictic at higher latitudes and tidal

elevations (i.e. parthenogenetic, Fierst *et al.* 2010). Therefore, at fine scales within the intertidal seascape, the same mechanisms as those which operate over a species' distributional range may be at work with core and marginal metapopulation dynamics. This necessitates the investigation of intertidal habitats to understand how tidal dynamics influence the partitioning of genetic structure and other intraspecific micro-evolutionary outcomes.

In addition to seascape features, biological characteristics of species are equally as important in understanding genetic structure at small scales. The algal seascape genetic literature is mainly composed of fucoid and kelp models which are not entirely representative of the extant life cycle diversity among seaweeds. The fucoid life cycle is composed of a single free-living diploid stage, similar to animal life cycles. The kelp life cycle alternates between a macroscopic sporophyte (diploid) and microscopic gametophytes (haploid), but the microscopic gametophyte stage is extremely difficult to study in the field. Therefore, studies of genetic structure in kelps use the macroscopic sporophyte stage and, as such, the genetic structure is the result of both zoospore and sperm dispersal, as in plants and animals. Yet, understanding the genetic structure of both the haploid and diploid free-living stages is important as haploid–diploid life cycles can significantly impact genetic structure and mating systems. If linked through sexual reproduction, haploid and diploid genomes are transmitted through each phase to the next. Genetic drift in the diploid subpopulation will affect the effective size of the haploid subpopulation and *vice versa*. In this context, red seaweeds are particularly tractable models for exploring the consequences of seascape features and biphasic life cycles on the spatial patterning of genetic structure.

The Florideophyte red algal life cycle is unique to this group of seaweeds. Nonmotile, very short-lived male gametes (spermatia) are released and fertilize an egg (carpogonium) which is retained on the female gametophytic thallus (Fig. 1). The zygote is mitotically amplified thousands of times within the cystocarp which is retained on the female thallus. This stage has been regarded as a possible mechanism compensating for the lack of flagellated sperm in red algae (Searles 1980). The resulting diploid carpospores are an additional dispersive stage in comparison with other algal life cycles and germinate into the free-living tetrasporophyte. The tetrasporophyte produces haploid tetraspores via meiosis which germinate into gametophytes (generally dioecious in most red algal species). The lack of motile propagules and three discrete dispersive agents probably generate distinct patterns of genetic structure in red seaweeds, highlighting the importance of investigating seascape genetics in conjunction with life history characteristics. First, as each carpospore produced within the same cystocarp is genetically identical, germination of many carpospores originating from the same cystocarp would lead to a reduction in overall population genetic diversity as many tetrasporophytes would share the same genotype. Second, if the diploid carpospores and haploid tetraspores have different dispersal capabilities, then

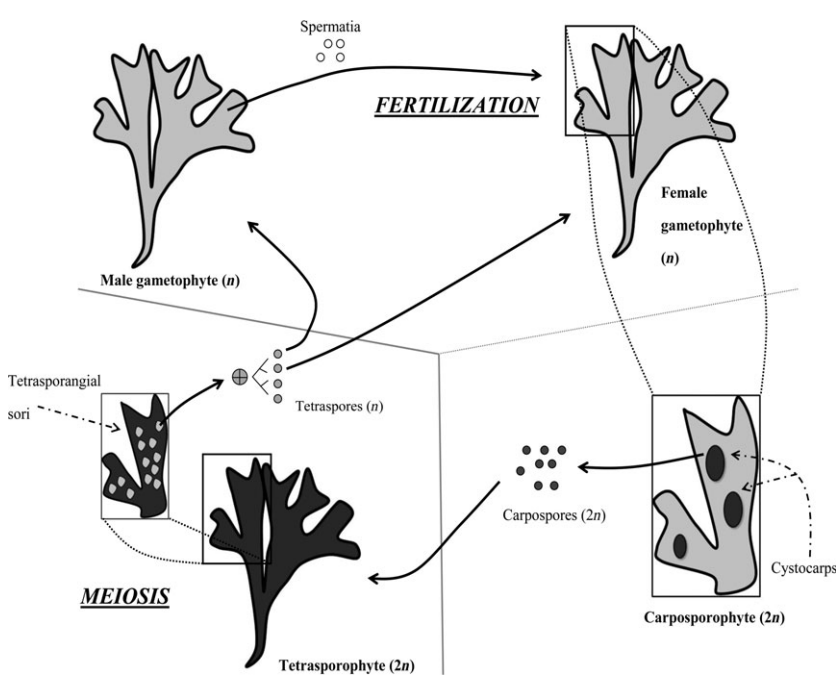

Fig. 1 The life cycle of *Chondrus crispus*, typical of the Florideophyceae. Nonmotile spermatia fertilize the carpogonium which is retained on the female gametophytic thallus (*fertilization*). Within the cystocarp, the zygote is mitotically amplified liberating thousands of diploid carpospores. The diploid carpospores produce the diploid free-living tetrasporophyte. *Meiosis* occurs in the tetrasporophyte releasing haploid tetraspores which form the free-living female and male gametophytes.

gene flow would be affected leading to different levels of genetic differentiation between the two life history stages. Third, if sexual reproduction is uncommon, then drift may lead to strong differences in allele frequencies between the haploid and diploid stages within the same life cycle. Finally, fourth, male gametes lack the ability to swim the final critical distance to the female gamete. Therefore, successful fertilization events may be rare (but see, Engel *et al.* 1999; Maggs *et al.* 2011).

Within the intertidal zone, both seascape (e.g. tidal cycles) and biological features (e.g. life history traits) govern genetic structure. Yet, this has rarely been explicitly studied (exceptions include Faugeron *et al.* 2001 and Engel *et al.* 2004). Engel *et al.* (2004) investigated the fine-scale genetic structure of both the haploid and diploid stages within tide pool populations of the red seaweed *Gracilaria gracilis*. Contrary to the expectation of predominantly endogamous mating systems in haploid–diploid organisms (Otto & Marks 1996), gamete unions in *G. gracilis* were found to occur in an allogamous manner (Engel *et al.* 1999, 2004). Weak, but significant population differentiation was detected and varied with seascape features and not with geographic distance, consistent with the results of most seascape genetic studies (e.g. White *et al.* 2010). Self-recruitment significantly increased with the duration of emersion time, reflecting the longer period of time, high shore pools were isolated at low tide and supporting predictions about the influence of tidal cycles.

In contrast to discrete tide pool populations, many seaweeds occur along a continuum of intertidal microsites from complete emersion to complete submersion at low tide, regardless of shore height. *Chondrus crispus* Stackhouse is an excellent example of a seaweed which forms dense stands within the mid-intertidal and into subtidal zones in the North Atlantic (Dixon & Irvine 1977). Barriers to gene flow in *C. crispus*, and therefore the effect of the intertidal seascape on genetic structure, may not be as immediately obvious as in tide pool populations of *G. gracilis*. Moreover, the dominance of gametophytes as well as the supposed rarity of male gametophytes poses interesting questions about the population structure (e.g. are tetrasporophytes competitively inferior?) and mating system in this species (e.g. how and what type of gamete unions occur if males are rare?).

We investigated the effects of tidal height on genetic variation in both gametophytic (haploid) and tetrasporophytic (diploid) stages of *C. crispus* using eight microsatellite loci to test the following hypotheses: (i) high and low shore individuals belong to genetically differentiated populations driven by tidal cycles of emersion, (ii) gene flow is restricted within high shore populations due to tidal-influenced isolation and marginality and (iii) previously reported large, significantly positive F_{IS} values are due to high levels of intergametophytic selfing (i.e. a specific type of inbreeding where mating occurs between two free-living gametophytes produced by the same tetrasporophyte, Klekowski 1969) driven by life history characteristics.

Materials and methods

Study species and genet definition

Chondrus crispus is economically and ecologically important in the North Atlantic. It follows an isomorphic haploid–diploid life history (Fig. 1; Chen & McLachlan 1972). In this study, free-living individuals which developed from different sexual events (zygote or spore) were considered genets. Consequently, individuals arising either from spores produced in the same cystocarp (i.e. a single fertilization event) or from two separate fertilization events with the same female–male pair were not considered genets even if morphologically distinct entities.

Study area and sampling design

A high shore and a low shore stand of *C. crispus* was sampled at the Port de Bloscon (48°73′N, 3°97′W) near the Station Biologique de Roscoff in Brittany, NW France to address whether: (i) high shore and low shore stands are genetically differentiated, (ii) genetic differentiation occurs within the high shore stand and (iii) the high levels of nonrandom mating detected are due to either inbreeding or population subdivision.

Temperature is one of the most important abiotic determinants of intertidal zonation (e.g. Pearson *et al.* 2009). Therefore, temperature loggers (ONSET Optic StowAway TidbiT Temp Logger; ONSET, Bourne, MA, USA) were installed within the high shore stand at ~3.6 m above mean low water (MLW) and within the low shore stand at ~2 m above MLW. Each logger was affixed to the substratum using a screw from July 2010 until July 2011 and was located within the *C. crispus* stand. Temperature data were recorded every 10 min. Therefore, depending on the tidal cycle, either ambient air or seawater temperature was recorded. This enabled the characterization of emersion time, based on the ambient temperature, in which the high shore stand was more often emerged and experienced higher temperatures as well as higher temperature variation as compared to the low shore stands (Fig. S1, Supporting information). In addition to differences in tidal height, the high shore and low shore stands were separated by ~20 m in horizontal topographical distance. At low tide, the high and low shore stands were separated by a large rocky prominence through which concentrated rivulets of water, and likely propagules, flow

unidirectionally from high to low. At high tide, the rocky prominence was completely submerged and did not cause a barrier to gene flow among stands located across the tidal range of *C. crispus*.

Two sampling strategies were adopted to explore the aforementioned hypotheses (Fig. 2). As it was not possible to sample the entire intertidal zone, the first strategy utilized a 90-m hierarchical transect, hereafter referred to as transect, parallel to the shore (Fig. 2b, c). These transects were sampled in both the high shore and low shore stands in April 2009. Based on previous results suggesting genets were globally <10 cm in diameter (Krueger-Hadfield *et al.* 2011), the minimum sampling distance of 25 cm was chosen to minimize the chances of sampling the same genet twice. A total of 15 fronds, if present, were sampled every 25 cm within a 1 m × 0.5 m quadrat (Fig. 2b). Each quadrat was nested within a pair separated by 0.5 m. Each pair was separated by 3 m and nested within a set of four. Each set of four was separated by 6 m to form a plot of eight quadrats. The plot of eight quadrats was repeated two more times along the shore, each separated by 12 m. Therefore, there were possible comparisons within plots (6 m), between plots 1 and 2 and 2 and 3 (12 m) and between plots 1 and 3 (46 m, Fig. 2c). This strategy enabled the determination of the distance at which spatial structuring occurred within each shore level and thus a representation of the genetic structure and mating system within the intertidal zone at the Port de Bloscon.

Second, as inbreeding and population subdivision could be due to limited dispersal leading to allelic correlation, the factors underlying nonrandom mating were explored using a different sampling strategy of continuous and evenly spaced points. This enabled spatial autocorrelation analyses to explore how the genetic resemblance between individuals varies with respect to the distance separating individuals (Hardy & Vekemans 1999). A single frond was sampled every 25 cm within a 5 m × 5 m grid, hereafter referred to as the 5-m grid, in February 2010 (Fig. 2d). A maximum sample size of 441 fronds was possible within the 5-m grid at each shore level. This strategy enabled an investigation of genetic structure and the mating system and estimates

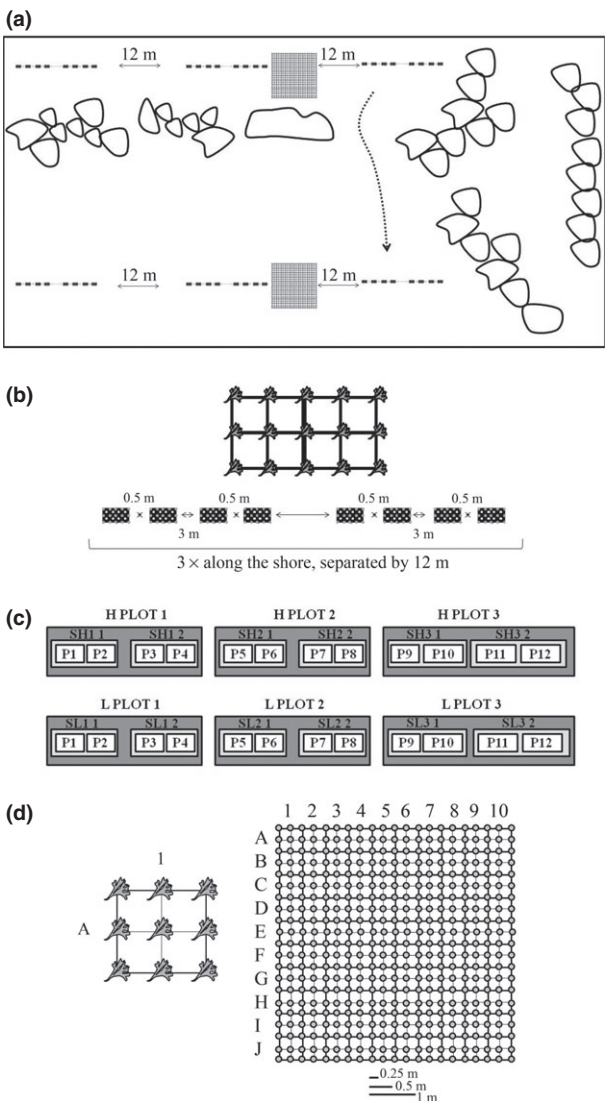

Fig. 2 (a) A schematic birds-eye view of the Port de Bloscon, near the Station Biologique de Roscoff, showing the approximate location of the 5-m grids, the transects and the boulder barrier. Distances are not shown to scale in the overall birds-eye view. (b) A schematic diagram of the hierarchical sampling methodology implemented along a 90-m transect high [3.61 m above MLW (mean low water)] and low on the shore (2.06 m above MLW) within the *Chondrus crispus* range. The smallest sampled scale was 1 m by 0.5 m quadrat, labelled as P1, P2, etc., in the schematic. Each of the quadrats were separated by different distances, with the group of eight quadrats forming 22 m of the transect. The 22-m group was repeated three times along the shore at both heights. (c) Hierarchical genetic differentiation of *C. crispus* gametophytes located in the high and low shore stands. F_{PQ} measures the differentiation between pairs of quadrats (e.g. P1 vs. P2). This was the smallest spatial scale with sufficient sample size to enable analyses. F_{SP} measures differentiation between sets of four quadrats (SH1 1 vs. SH1 2: 6 m). Finally, F_{ST} measures differentiation between plots of 8 quadrats (H PLOT 1 vs. H PLOT 2: 12 m; H PLOT 1 vs. H PLOT 3: 46 m). Estimates of pairwise differentiation were obtained from within stands (i.e. high vs. high and low vs. low) and between stands (i.e. high vs. low). (d) The high 5-m grid (3.61 m above MLW) and the low 5-m grid (2.06 m above MLW) were composed of continuous and evenly spaced points, where a single frond from each genet (dots) was sampled, if present, every 25 cm. In the field, each square was the equivalent of four 0.25 m^2 quadrats. MLW, mean low water.

of density and ploidy ratios within a 25 m^2 patch of each shore level.

Identification of life history stage and sex ratios

Female gametophytes (haploid), male gametophytes (haploid) and tetrasporophytes (diploid) were identified by their reproductive organs: (i) fertilized female gametophytes identified by the presence of cystocarps, (ii) reproductive male gametophytes identified by a pinkish to white band of spermatangial sori 3–10 mm below the apex (Tveter-Gallgher *et al.* 1980) and (iii) reproductive tetrasporophytes by the presence of tetrasporangial sori. Male gametophytes were not phenotypically identified for the transects. To determine the ploidy level of vegetative fronds, the acetal-resorcinol reaction colour test modified by Lazo *et al.* (1989) was used.

The binomial law was used to estimate the probability of detecting haploid to diploid ratios (including both reproductive and vegetative plants) deviating from the null hypothesis of the gametophyte to tetrasporophyte (G:T) ratio of $\sqrt{2}$:1. Indeed, $\sqrt{2}$:1 (and not 1:1) is the expected ratio at demographic equilibrium when haploids and diploids are equal in fitness and sex ratios are equal to 1:1 (Destombe *et al.* 1989; Thorber & Gaines 2004). All tetrasporophytes produce males and females via meiosis, while only female gametophytes produce offspring. The binomial law was also used to detect sex ratios deviating from the null hypothesis of female to male ratios of 1:1 (Fisher 1930). The sex ratios could only be tested with reproductive gametophytes.

DNA extraction and microsatellite amplification

Total genomic DNA was extracted using the Nucleospin® 96 plant kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instruction except for the cell lysis buffer in which samples were left at room temperature for one hour rather than heating to 65 °C for 30 min. Five to 10 mg of dried tissue was used for each sample and eluted in 100 μL elution buffer.

Five new, polymorphic microsatellites were added to the three most polymorphic microsatellites from Krueger-Hadfield *et al.* (2011; Table 1). The new microsatellites were developed from genomic sequences following the same protocol previously described (Krueger-Hadfield *et al.* 2011).

Multiplex PCRs were performed, amplifying several loci simultaneously using a PT-200 thermocycler (MJ Research, Waltham, MA, USA): 20 μL final volume, 5 μL of DNA template, 1× buffer, 150 μM dNTP, 2 mM $MgCl_2$, varying concentrations of primers (see below; the forward primer was fluorescently labelled) and 0.35 U taq polymerase (GoTaq Flexi; Promega) per primer pair in the reaction. Primer concentrations were experimentally determined such that the intensity of all microsatellites was high enough to prevent allelic dropout and allow unambiguous genotyping. For multiplex 1, primer concentrations were 0.2 μM for Chc_24 and Chc_31, 0.3 μM for Chc_40 and 0.4 μM for Chc_23. For multiplex 2, primer concentrations were 0.3 μM for Chc_03 and Chc_35 and 0.4 μM for Chc_02 and Chc_04. The PCR programme included: 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at either 60 °C (multiplex 1) or 55 °C (multiplex 2), 30 s at 72 °C; and finally, 10 min at 72 °C. Two microlitre of each PCR product, diluted to 1:10, was added to 10 μL of loading buffer containing 0.3 μL of size standard (GeneScan—600 Liz; Applied Biosystems, Foster City, CA, USA) plus 9.7 μL of Hi-Di formamide (Applied Biosystems). The loading mix was denatured at 92 °C for 3 min and run in an ABI 3130 xl capillary sequencer (Applied Biosystems) equipped with 50-cm capillaries.

Genotypes were scored manually using GENEMAPPER ver. 4 (Applied Biosystems). Automated binning, using manually defined bins in GENEMAPPER, resulted in each locus exhibiting a large amount of even and odd alleles in *C. crispus*. Therefore, alleles were binned using the program TANDEM (Matschiner & Salzburger 2009). Multilocus genotypes (MLG) were then compared to the results from the resorcinol tests to verify all gametophytes displayed one allele per locus and tetrasporophytes displayed one or two alleles per locus (i.e. homozygotes or heterozygotes).

Microsatellite data analyses

For each of the following analyses, the 5-m grids and transects were treated separately. Prior to analyses, the number of repeated identical multilocus microsatellite genotypes was computed using the Mutlilocus Matches option in GENALEX, ver. 6.41 (Peakall & Smouse 2006). This option automates detection of repeated genotypes within the data set. Repeated MLG may occur due to one or more of the following: (i) repeated sampling of the same genet (i.e. some genets may be larger than 10 cm in diameter), (ii) asexual production of gametes or spores, (iii) breakage of one genet into two or more thalli due to perturbation (e.g. disturbance, herbivory or partial mortality of the holdfast, or the structure which anchors the alga to the substratum), (iv) production of genetically identical carpospores within the cystocarp, (v) production of spores from two different cystocarps produced from fertilization events of the same male–female pair or (vi) two distinct sexual events wherein the offspring share the exact same alleles at all loci, but not the same male–female pair. The frequency of different MLG was calculated

Table 1 Gene and allelic diversities of eight polymorphic microsatellite markers isolated from a high and a low shore stand of *Chondrus crispus* according to ploidy level in (a) the 5 m × 5 m grids and (b) the transects at the Port de Bloscon

(a)

			High								Low							
			Diploid N = 57				Haploid N = 115			Δ	Diploid N = 72				Haploid N = 228			Δ
Locus	Acc. no.	H_T	N_{all}	A_E	H_E	F_{IS}	N_{all}	A_E	H_E^a	P-value	N_{all}	A_E	H_E	F_{IS}	N_{all}	A_E	H_E^a	P-value
Chc_02	HM444812	0.760	12	12.0	0.763	0.304*	13	12.1	0.735	0.313	17	16.0	0.750	0.161*	17	14.4	0.685	0.041
Chc_03	HM444813	0.866	15	14.9	0.801	0.337*	16	14.9	0.787	0.256	18	16.9	0.882	0.112*	24	17.7	0.894	0.177
Chc_04	HM444814	0.887	20	19.9	0.867	0.490*	23	20.4	0.915	0.386	30	26.1	0.897	0.267*	43	29.3	0.932	0.322
Chc_23	KC188839	0.985	49	48.6	0.972	0.472*	54	45.5	0.979	0.019	59	53.8	0.982	0.379*	89	58.8	0.989	***0.005***
Chc_24	KC188840	0.952	31	30.9	0.905	0.413*	32	27.7	0.891	0.398	44	41.0	0.972	0.151*	50	37.4	0.954	***0.003***
Chc_31	KC188842	0.918	19	18.9	0.894	0.406*	21	19.2	0.922	0.042	24	22.3	0.913	0.142*	23	18.2	0.899	0.100
Chc_35	KC188843	0.942	25	24.9	0.913	0.380*	26	23.8	0.944	**8.4×10^{-4}**	26	25.0	0.950	0.220*	31	24.7	0.944	0.723
Chc_40	KC188841	0.972	37	36.7	0.967	0.304*	37	32.8	0.965	0.093	43	40.2	0.973	0.152*	56	42.6	0.978	0.398
Mean over loci		0.910	26	25.8	0.885	0.398*	27.8	24.5	0.888	**4.1×10^{-4}**	32.6	30.2	0.915	0.207*	41.6	30.4	0.907	**4.5×10^{-4}**
SE		0.026	4.4	4.0	0.026		4.7	3.6	0.031		5.2	4.6	0.027		8.3	5.6	0.034	

(b)

			High								Low							
			Diploid N = 52				Haploid N = 92			Δ	Diploid N = 32				Haploid N = 151			Δ
Locus	Acc. no.	H_T	N_{all}	A_E	H_E	F_{IS}	N_{all}	A_E	H_E^a	P-value	N_{all}	A_E	H_E	F_{IS}	N_{all}	A_E	H_E^a	P-value
Chc_02	HM444812	0.972	13	11.3	0.839	0.268*	15	12.4	0.822	0.232	13	12.3	0.731	0.218*	17	13.1	0.807	0.083
Chc_03	HM444813	0.931	14	11.1	0.832	0.183*	14	10.7	0.816	0.478	18	17.4	0.915	0.069	17	12.9	0.881	0.369
Chc_04	HM444814	0.974	18	14.1	0.849	0.310*	23	16.0	0.881	0.701	19	19	0.934	0.515*	41	23.3	0.944	0.097
Chc_23	KC188839	0.938	34	26.8	0.956	0.626*	52	33.3	0.987	**0.002**	25	24.1	0.964	0.796*	69	34.5	0.985	0.017
Chc_24	KC188840	0.926	33	23.7	0.895	0.262*	37	24.7	0.952	0.382	29	26.6	0.955	0.202*	50	28.5	0.968	0.079
Chc_31	KC188842	0.902	24	19.4	0.924	0.223*	23	17.6	0.935	0.041	18	16.7	0.897	0.186*	28	18.2	0.929	0.468
Chc_35	KC188843	0.876	17	14.9	0.903	0.496*	20	16.7	0.944	0.381	20	19.3	0.947	0.430*	29	20.3	0.952	0.509
Chc_40	KC188841	0.805	34	25.8	0.963	0.321*	37	26.2	0.973	0.431	30	28.1	0.965	0.243*	50	30.5	0.981	0.019
Mean over loci		0.916	23.4	18.4	0.895	0.349*	27.6	19.7	0.909	0.020	21.5	20.4	0.913	0.353*	37.6	22.7	0.928	***0.003***
SE		0.018	3.2	2.3	0.018		4.7	2.7	0.023		2.1	1.9	0.027		6.4	2.8	0.021	

H_T, total heterozygosity over whole sample of tetrasporophytes (n = 129); N, number of sampled individuals, note that for the diploids, 2N genes were sampled; N_{all}, A_E, H_E, number of alleles, estimates of expected allelic richness based on the smallest sample size (5 m × 5 m populations: 112 genes; transects: 64 genes), expected heterozygosities at the population level; H_E^a, in haploids, H_E was corrected by a factor of $(2N-1)/(2N-2)$; F_{IS}, fixation index and test for deviation from Hardy–Weinberg expectations were performed using permutation tests (*$P < 0.001$) for diploid subpopulations only; Δ, differences in allelic frequencies between haploids and diploids of the same population; P-value, probability associated with Fisher's exact test combined across loci on contingency tables of observed haploid and diploid allele distributions. P-values highlighted in bold were significant (Bonferroni-corrected threshold P-value = 0.002 for $\alpha = 0.05$) in bold and italics were near the threshold.

independently in the haploid gametophytes and diploid tetrasporophytes as follows:

$$D_{\text{haploid}} = \text{MLGH}/N_{\text{haploid}},$$

$$D_{\text{diploid}} = \text{MLGD}/N_{\text{diploid}},$$

where *MLGH* is the number of distinct haploid MLG, *MLGD* is the number of distinct diploid MLG and *N* is the total number of studied individuals, haploid or diploid (Ellstrand & Roose 1987 modified by Guillemin *et al.* 2008). To estimate whether putative genets shared the same MLG, GENCLONE 2.0 was used (Arnaud-Haond & Belkhir 2007). For each gametophyte or tetrasporophyte, P_{sex}, which is the probability for a given multilocus genotype to be observed in *N* samples as a consequence of different sexual reproductive events, was calculated for each repeated MLG. If P_{sex} was >0.05, duplicated MLG were considered as different genets. If P_{sex} was smaller than 0.05, the duplicated MLG were considered as ramets (or clones) of the same genet. As P_{sex} was always found to be <0.05, only one genotype per repeated MLG was retained in subsequent analyses to avoid biases in different estimates.

The frequency of null alleles was directly estimated for the haploid gametophytes from individuals in which there was no amplified PCR product after discounting technical errors. The frequency of null alleles in the tetrasporophytes was calculated using a maximum-likelihood estimator in the software ML-NULLFREQ (Kalinowski & Taper 2006). Linkage disequilibrium was tested separately in the gametophytes and tetrasporophytes using GENEPOP, ver. 4.1 (Rousset 2008) to further describe the mating system and spatial structure. In addition to physical linkage on a chromosome, disequilibria may be due to a lack of recombination caused by clonal propagation or selfing (mating system) or to differences in allele frequencies among populations (spatial genetic structure). Significance testing was performed using 1000 permutations and Bonferroni correction (Sokal & Rohlf 1995).

For each population, the mean number of alleles per locus (A_{O}) and the average expected heterozygosity (H_{E}) were calculated for both the gametophytes and the tetrasporophytes using GENALEX. The haploid and diploid stages are expected to show similar genetic diversities and allele frequencies in the absence of asexual reproduction or differential selection (Halkett *et al.* 2005). The rarefaction method of El Mousadik & Petit (1996), modified by Engel *et al.* (2004) for haploid–diploid species, was used to correct for the disparity in the number of genes in haploids and diploids. An estimate of the mean expected number of alleles (A_{E}) in a sample containing n_i genes given that $2N_i$ genes have been sampled (where n_i is the number of sampled haploid individuals and N_i is the number of sampled diploid individuals in population *i*) was computed using the program HP_RARE (Kalinowski 2005). Exact tests for differences in allele frequencies between ploidy levels within each population were performed on the distribution of allelic counts using the program GENEPOP, ver. 4.1 (Rousset 2008).

Three-way analysis of variance (ANOVA) was performed to test the effects of ploidy, population and locus on H_{E} and A_{E}. Genetic diversity is hypothesized to be the same in each ploidy level due to the regular occurrence of sexual reproduction, whereas genetic diversity is expected to be lower in high shore vs. low shore stands due to increasing marginality higher on the shore. As each locus varies in terms of polymorphism, H_{E} and A_{E} are expected to vary by locus. Ploidy and population were declared fixed factors, whereas locus was declared random. General linear model procedures were used as implemented in MINITAB ver. 15 (State College, PA, USA).

Tests for Hardy–Weinberg equilibrium and *F*-statistics were performed on diploid tetrasporophytes using FSTAT, ver. 2.9.3.2 (Goudet 1995). F_{IS} was calculated for each locus and over all loci according to Weir & Cockerham (1984), and significance was tested by running 1000 permutations of alleles among individuals within samples. To test the hypothesis that high and low shore stands are genetically differentiated from one another, pairwise F_{ST}s were calculated between the high and low shore 5-m grids using GENEPOP, ver. 4.1 (Rousset 2008) in the gametophytes and tetrasporophtyes separately. Genetic differentiation was also estimated at different spatial scales of hierarchical sampling using GENEPOP (Fig. 2). These analyses enabled both within-stand and between-stand genetic differentiation characterizations at three spatial scales to test the aforementioned hypotheses (Fig. 2c). At the largest scale, pairwise F_{ST} was calculated between all pairs of transect plots (i.e. each group of eight quadrats replicated three times parallel to the shore within each stand) in the gametophytes and tetrasporophytes separately. Nested within plots, F_{SP} measured differentiation between sets of four quadrats. F_{PQ} measured the differentiation between pairs of quadrats. This was the smallest spatial scale with sufficient sample size to enable analyses. However, due to the paucity of tetrasporophytes, F_{SP} and F_{PQ} were measured in the gametophytes only. For F_{SP} and F_{PQ}, there were several values from which a mean and a standard deviation were calculated.

The program SPAGEDI, ver. 1.3 was used to determine whether the populations were subdivided, generating large F_{IS} values (Hardy & Vekemans 2002). Ritland's (1996) kinship coefficients for each distance class and

regressed on pairwise separation distance. Within each population, gametophytes and tetrasporophytes were analysed together. However, haploids and diploids may have different dispersal characteristics, so the spatial structure was analysed separately for each life history phase as well. Due to the paucity of tetrasporophytes in the two transects, spatial autocorrelation was performed on each transect as a whole (both gametophytes and tetrasporophtyes) and for the gametophtyes alone. Distance classes were chosen to optimize the number of comparisons for each distance class. Significance testing was performed by comparing the observed pairwise coefficients with the corresponding frequency distributions of 1000 random permutations.

To determine whether the high and low shore stands could be characterized as two genetically differentiated populations, a Bayesian method to identify clusters of genetically similar individuals was used. In the program STRUCTURE, clusters are assigned based on individual MLG by creating groups within which Hardy–Weinberg and linkage disequilibrium are minimized (Pritchard *et al.* 2000). After inferring the most likely number of different clusters, the ancestry coefficient of each individual is estimated. Yet, inbreeding may induce linkage disequilibrium among loci and Hardy–Weinberg disequilibrium which can pose problems for clustering analyses. *Chondrus crispus* populations previously studied were thought to be either spatially subdivided or subject to high levels of inbreeding (Krueger-Hadfield *et al.* 2011). Gao *et al.* (2007) developed the software INSTRUCT which takes into account the possibility of selfing by eliminating the assumption of Hardy–Weinberg equilibrium within clusters. Simulations were performed on the diploid tetrasporophytes sampled in the 5-m grids to estimate structure with a model including both bi-parental inbreeding and admixture, where each individual draws some fraction of its genome from each of the K populations. A burn-in of 300 000 repetitions and a run length of 500 000 were used for $K = 1$ to $K = 6$, where 10 iterations for each K were run. INSTRUCT chooses the value of K favoured by the deviance information criteria (DIC), which implies the model assuming the optimal K value which fits the data set most. However, selecting $K = 6$, $K = 7$, etc., resulted in the largest possible K chosen by the DIC. Therefore, $K = 1$ to $K = 15$ was tested to determine the optimal K, according to INSTRUCT. K was determined by the method developed by Evanno *et al.* (2005) as DIC tends to overestimate the most informative number of clusters. To ensure biological relevance, this value was then compared to the K which consistently produced the same result. The estimated cluster membership coefficient (Q) matrices for INSTRUCT were graphically displayed using DISTRUCT, ver. 1.1 (Rosenberg 2004).

Results

Life history and sex ratio

With the exception of the high shore transect ($P = 0.134$), all populations were significantly gametophyte-biased, differing from the expected gametophyte to tetrasporophyte ratio of $\sqrt{2}$:1 (each $P \leq 0.016$; Fig. 3). However, the magnitude of gametophyte-bias was reduced in the high shore 5-m grid. Both high and low shore 5-m grids were also significantly female-biased (each $P \leq 0.005$; Fig. 3). The high shore was also characterized by reduced frond density in comparison with the low shore stand (Fig. S2, Supporting information).

Microsatellite allele binning

The authors of TANDEM suggest that good loci have an average rounding error which is below 10% of the repeat size (Matschiner & Salzburger 2009). Loci Chc_02, Chc_03, Chc_23 and Chc_35 exhibited rounding errors above 0.2 (data not shown). TANDEM outputs the individuals in which alleles may have been poorly binned. For each poorly binned individual, the true allele size (i.e. allele size to two decimal places) was compared to individuals in the data set with similar true allele sizes. Each of the potentially problematic individuals was rounded into the same bin as nonproblematic individuals and therefore did not artificially over- or underestimate allelic richness. Moreover, there were no consistent patterns between *good* or *bad* loci in downstream analyses which might indicate a problematic locus (see also Pennings *et al.* 2011). Therefore, all eight loci, including Chc_02, Chc_03, Chc_23 and Chc_35, were retained.

Multilocus genotype analyses, null alleles and linkage disequilibria

Two pairs of gametophytes from the low shore 5-m grid and two pairs of gametophytes from the low transect shared the same multilocus genotype (MLG). Each frond from these pairs of gametophytes was located within 25 cm of the other frond. There were no tetrasporophytes which shared the same MLG. The values of D_{haploid} ranged from 0.987 to 1.0, indicating almost all genotypes sampled were different. The P_{sex} values were all considerably smaller than 0.05 (i.e. each value $<5.0 \times 10^{-7}$) suggesting each repeated MLG was most likely re-sampling of the same genet. There were no repeated tetrasporophytic MLGs, and D_{diploid} was equivalent to 1 for the 5-m grid and transect in each shore level.

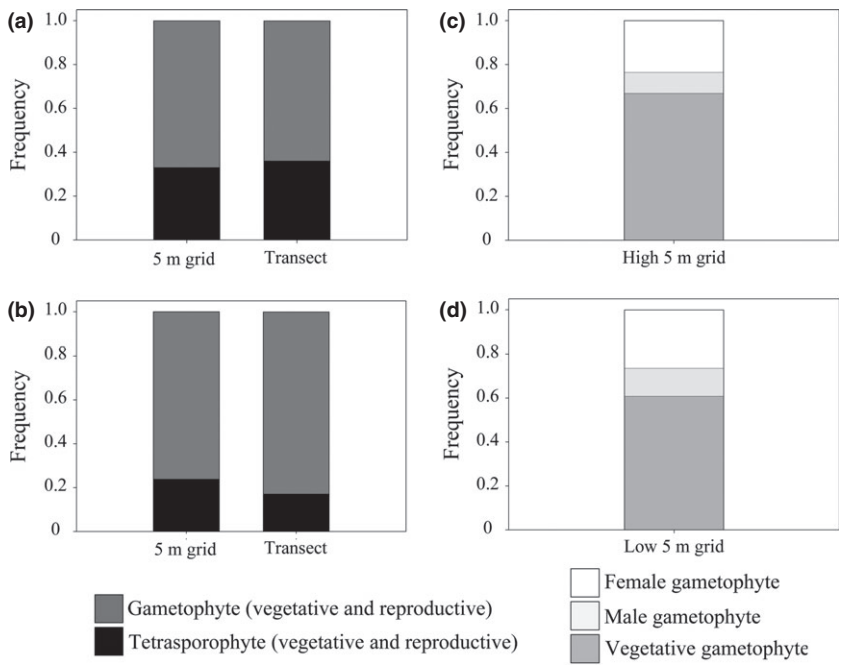

Fig. 3 The total number of vegetative and reproductive gametophytes and tetrasporophytes sampled at the Port de Bloscon (a) high 5-m grid ($P < 0.05$) and transect ($P > 0.05$, NS) and (b) low 5-m grid ($P < 0.001$) and transect ($P < 0.001$). The total number of vegetative gametophytes, reproductive female and male gametophytes (c) in the high 5-m grid ($P < 0.001$) and d) low 5-m grid ($P < 0.001$).

As both diploids and haploids were studied, it was possible to compare the frequency of null alleles estimated directly in the haploid gametophytes (i.e. individuals in which there was no amplification) with the null allele estimates in the diploid tetrasporophytes using maximum likelihood. There were discrepancies between the two estimates of null allele frequencies. Generally, there were no null alleles detected in the gametophytes in the 5-m grids, whereas, in the transects, there were null alleles detected at each locus, although the frequencies were <0.043 (Table S1, Supporting information). In contrast, the frequency of null alleles detected in the tetrasporophytes varied from at least 0.050 to 0.408 (Table S1, Supporting information).

Overall, there was little evidence of linkage disequilibrium between pairs of loci after Bonferroni correction in each of the ploidies within the 5-m grids and transects.

Genetic diversity, mating system and genetic differentiation

The expected heterozygosities (H_E) within subpopulations were high (>0.7 in average) and varied locus by locus (Table 1). H_E was similar between the gametophytes and tetrasporophtyes ($0.108 < P < 0.939$; Table S2, Supporting information). When adjusted to the smallest sample size (A_E), the numbers of alleles were not different between the tetrasporophytes and gametophytes ($0.051 < P < 0.565$; Table S2, Supporting information). There was a significant difference between estimates of H_E between the high and low shore stand 5-m grids ($P < 0.025$), but not the transects ($P = 0.113$, Table S2, Supporting information). The level of polymorphism varied between loci (all $P < 0.001$), where loci Chc_23, Chc_24 and Chc_40 were the most polymorphic.

In the 5-m grids, there was significant differentiation (after Bonferroni correction) in allele frequencies between the gametophytes and tetrasporophytes (both $P = 4 \times 10^{-4}$; Table 1). In the transects, the P-value in the low shore stand was close to significant ($P = 0.003$), whereas there was no difference in allele frequencies in the high shore stand after Bonferroni correction (Table 1). When locus Chc_23 was removed, there were no longer differences in allele frequencies (data not shown). All except one single-locus F_{IS} estimates were positive and significantly different from zero as well as all the multilocus F_{IS} estimates for each of the populations (Table 1). Therefore, the intertidal shore sampled was significantly heterozygote deficient. F_{IS} values were larger in the high shore 5-m grid ($F_{IS} = 0.398$) compared with the low shore 5-m grid ($F_{IS} = 0.207$), whereas there were no differences between transects (high $F_{IS} = 0.349$ vs. low $F_{IS} = 0.353$). Jackknife standard errors, as provided in FSTAT, were small for both the 5-m grids (SE = 0.027) and transects (SE = 0.018), verifying congruence among loci.

There was little evidence of spatial structure as few spatial genetic structure regression slopes were significant in the 5-m grids and along transects (Table 2). In the high 5-m grid and along the high transect, when the sample size was sufficient, there were significant positive kinship coefficients, especially at 25 cm (Fig. S4, Supporting information). Related individuals were grouped together and beyond 1 m the kinship

Table 2 Slopes of the correlograms obtained from each locus and multilocus estimates from each 5 m × 5 m grid and transect over the distance classes <6 or <1 m using the kinship coefficient proposed by Ritland (1996) as calculated using the program SPAGEDI

	Low			High		
Locus	*H*	*D*	Both	*H*	*D*	Both
<6 m 5 m × 5 m	*-3.44×10^{-4}* (228)	**7.74×10^{-4}** (72)	-1.88×10^{-4} (300)	-6.91×10^{-6} (115)	7.02×10^{-4} (57)	6.15×10^{-5} (172)
<1 m 5 m × 5 m	−0.005 (228)	−0.008 (72)	−0.004 (300)	**−0.033** (115)	−0.003 (57)	**−0.012** (172)
<6-m transect	3.67×10^{-06} (151)	—	-1.37×10^{-05} (183)	-4.42×10^{-05} (92)	—	-1.52×10^{-05} (144)
<1-m transect	−0.007 (151)	—	6.30×10^{-05} (183)	*−0.027* (92)	—	−0.007 (144)

Significant slopes shown in bold and close to significant slopes ($0.05 > P < 0.07$) shown in italics; samples sizes shown in parentheses.

coefficients decreased with increasing distance until individuals were not significantly different from random (Fig. S4, Supporting information). In the low 5 m, there were no positive kinship coefficients, but there was still a pattern of decreasing kinship coefficients with increasing distance >1 m (Fig. S4, Supporting information). Spatial autocorrelation analyses were re-run for each 5-m grid and transect and each ploidy (except tetrasporophytes along the transects due to insufficient sample size) over the distance classes 0.25–1 m (Fig. S4, Supporting information). In the high shore, the slope was significant when observing the kinship coefficients every 25 cm between a reduced distance of 25 cm–1 m, suggesting that related individuals were grouped together under 1 m (Fig. S4, Supporting information, Table 2). In contrast, there was no such structure in the low shore nor were the slopes significant (Table 2).

Analyses using INSTRUCT identified two clusters of individuals corresponding to high and low shore stands (Fig. 4). At values $K = 3$ and $K = 4$, additional clusters appeared which did not correspond to specific shore levels, but instead indicated genetic heterogeneity within shore levels (not shown). The optimal number of clusters, as determined by DIC, was $K = 8$. However, at these values of K, the effectiveness of INSTRUCT diminished as the subdivisions became finer and the program identified each inbred line as a separate population (see Tatarenkov *et al.* 2007 or Ness *et al.* 2010). $K = 2$ was the value of K indicated by the method of Evanno *et al.* (2005) and showed consistent results for each independent run (data not shown).

Differentiation between the high and low shore was significant regardless the distance between plots, where the distance between high and low shore plots was always at least 20 m (Table 3a). Similarly, significant differentiation was found between the high and low shore 5-m grid haploids ($F_{ST} = 0.019$) and diploids ($F_{ST} = 0.018$). Significant spatial sub-structuring was only observed high on the shore and at distances >46 m

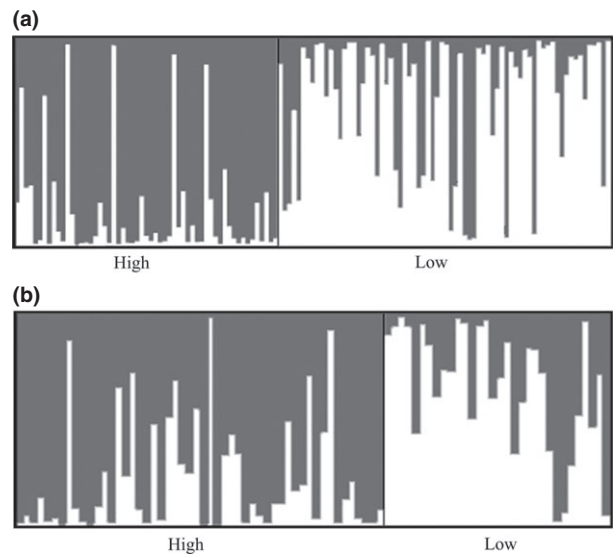

Fig. 4 The genetic structure of the (a) 5-m grids and (b) transects at both shore levels at the Port de Bloscon inferred from cluster analyses using InStruct. Each thin bar represents a single individual, which may be partitioned into K colours depending on the estimated multilocus membership in each of K clusters, where each colour represents the posterior probability of that individual belonging to a cluster. The best fitting cluster for each sampling strategy was $K = 2$.

(Table 3b, c). In contrast, there was no sub-structuring observed low on the shore (Table 3b, c). Finally, jackknife standard errors, as provided by FSTAT, were small for both the 5-m grids (SE = 0.029) and transects (SE = 0.018) verifying congruence among loci and although small, the F_{ST} estimates were significant.

Discussion

By integrating genetic structure with the intertidal seascape, new insights can be gained into connectivity patterns which are biologically relevant, even over very small scales. Microgeographic genetic differentiation

Table 3 (a) Pairwise estimates of genetic differentiation (F_{ST}) between plots of eight quadrats of *Chondrus crispus* along the transects of gametophytes (shown above diagonal and in italics) and tetrasporophytes (shown below diagonal) at both shore levels at the Port de Bloscon. For example, between-stand comparisons occur between H1 and L1 and within-stand comparisons occur between H1 and H2. The gametophyte sample size for each plot was the following: H1: n = 31; H2: n = 30; H3: n = 25; L1: n = 47; L2: n = 62; L3: n = 42. The tetrasporophyte sample size for each plot was the following: H1: n = 26; H2: n = 12; H3: n = 14; L1: n = 6; L2: n = 14; L3: n = 12. *P-values of <0.05 and marginally significant and ***P-values of <0.001 (Bonferroni-corrected threshold P-value = 0.03 for α = 0.05). (b) The average of the estimates of genetic differentiation (F_{SP}) between the sets of 4 quadrats along the transects of gametophytes within the high shore, within the low shore and between shore levels shown as mean ± standard deviation. Averages were calculated between F_{SP} estimates within plots (i.e. SH1 1 vs. SH1 2, 6 m), between plots 1 and 2 and 2 and 3 (i.e. SH1 2 vs. SH2 1, 12 m) and between plots 1 and 3 (i.e. SH1 2 vs. SH3 1, 46 m). (c) The average of the estimates of genetic differentiation (F_{PQ}) between pairs of two quadrats along the transects of gametophytes within the high shore and within the low shore shown as mean ± standard deviation. Averages were calculated between F_{PQ} estimates between quadrat pairs (i.e. P1 vs. P2), within plots (i.e. P1 vs. P3), between plots 1 and 2 and 2 and 3 (i.e. P4 vs. P5) and between plots 1 and 3 (i.e. P4 vs. P9)

(a)

	H1	H2	H3	L1	L2	L3
H1		*0.006*	*0.010**	*0.008**	*0.011**	*0.006*
H2	0.005		*−0.002*	*0.007**	*0.008**	*0.011****
H3	−0.005	0.012		*0.001*	*−0.001*	*0.004*
L1	0.030***	0.032***	0.022***		*−0.003*	*−0.004*
L2	0.023***	0.022***	0.011	0.022		*−0.003*
L3	0.010	0.014	0.008	0.010	0.001	

(b)

	High shore	Low shore	High shore vs. low shore
Within plots	0.003 ± 0.007	−0.001 ± 0.007	0.006 ± 0.005
Between plots (1 vs. 2 and 2 vs. 3)	0.006 ± 0.008	−0.003 ± 0.003	0.007 ± 0.008
Between plots (1 vs. 3)	0.011 ± 0.007	−0.005 ± 0.004	0.003 ± 0.006

(c)

	High shore	Low shore
Between pairs	0.007 ± 0.020	0.009 ± 0.015
Within plots	0.007 ± 0.018	0.002 ± 0.013
Between plots (1 vs. 2 and 2 vs. 3)	0.010 ± 0.023	0.001 ± 0.010
Between plots (1 vs. 3)	0.026 ± 0.034	−0.003 ± 0.012

*$P < 0.05$; ***$P < 0.001$.

within a single shore is therefore possible due to the small-scale patchiness of habitat and the spatiotemporal isolation of high shore populations. The high shore stand was characterized by lower genetic diversity accompanied by higher levels of intergametophytic selfing in comparison with the low shore stand. As significant spatial structure and lower levels of genetic diversity are signs of relative isolation, the high shore *Chondrus crispus* stand was more isolated than the low shore stand. In conjunction with the significant differentiation between shore levels, these results suggest an effect of intertidal seascape, specifically tidal-influenced isolation, on genetic structure in this seaweed.

The genetic diversity (H_E) exhibited in the high shore and low shore stands of *C. crispus* was among the highest values observed using microsatellite loci in seaweeds (see for example Valero *et al.* 2011 for a recent review in kelps and for reds: Engel *et al.* 2004; Guillemin *et al.* 2008; Couceiro *et al.* 2011). Higher genetic diversity is likely a result of higher mutation rates and larger effective population sizes in *C. crispus*. As the genome has been sequenced (J. Collén, personal communication), it was possible to be more selective and choose the most variable loci in comparison with other species for which there is no genomic information. The results pertaining to reproductive mode, mating system, spatial structure and genetic differentiation in *C. crispus* are discussed in the light of the haploid–diploid life cycle and the intertidal seascape.

Sexual reproduction and algal genets

Recruitment of individuals into the studied stands of *C. crispus* was likely the result of sexual reproduction (i.e. the settlement of sexually produced spores). Asexual reproduction leaves genetic signatures, such as

linkage disequilibria, differences in allele frequencies between life history stages, heterozygote excess and repeated MLGs (Guillemin *et al.* 2008). Yet, in *C. crispus*, there was an absence of significant linkage disequilibrium, globally no differences in allele frequencies between the haploids and diploids, strong heterozygote deficiency and few repeated MLGs ($0.98 \leq D \leq 1$), suggesting sexual reproduction. The frequency of repeated MLGs was much lower than values found in other seaweeds, such as in natural (mean $D = 0.6$) and farmed populations (mean $D = 0.3$) of the clonally reproducing red seaweed *Gracilaria chilensis* (Guillemin *et al.* 2008), whereas values were similar to those found in the sexually reproducing *Gracilaria gracilis* (mean $D = 0.94$, Engel *et al.* 2004). The few repeated gametophytic MLGs were located within 50 cm of each other and most likely represented sampling the same genet twice.

There were no repeated tetrasporophytic MLGs, suggesting that mitotic (clonal) amplification of the zygote within a cystocarp had no effect on population structure. If regular germination of diploid spores originating from the same cystocarp or from the same female–male pair were occurring, then one would expect to detect a cystocarpic signature in the population. For example, in *G. gracilis*, there were diploid MLGs shared by at least two individuals, with one diploid MLG shared by five individuals (Engel *et al.* 2004). In *C. crispus*, the lack of repeated diploid MLGs might be due to the sampling methodology or the real absence of diploid MLGs. All signs of repeated diploid MLGs might be lost by sampling at a scale of 25 cm, especially if spores recruit close to one another. However, the sampling strategy employed in this study might not be the underlying factor. Instead, cystocarpic amplification might result in clumped diploid spore recruitment and germination, where the cystocarpic signature is localized to a few centimetres. Yet, it would be impossible to determine whether an adult tetrasporophyte is the product of the germination of a single spore or many spores as all the spores share the same genotype.

Sampling a single frond did not provide any information about the size or composition of *C. crispus* holdfasts. Laboratory and field analyses of holdfast composition have revealed mosaics of not only different genotypes (i.e. different genets which appear to share the same holdfast), but also different ploidies (S. A. Krueger-Hadfield, C. Destombe & M. Valero, unpublished data). Thus, there is likely a high frequency of holdfast coalescence in the field (see also, Plumb 1999). However, there was no evidence of genetic chimeras in any of the fronds genotyped (including Krueger-Hadfield *et al.* 2011 and unpublished data). This suggests, although holdfasts may appear to be coalesced, there is no merging of genetic material between genets. Indeed, Tveter & Mathieson (1976) found coalesced juvenile gametophytes exhibit distinct sporeling (i.e. genotype and/or sex) delineation and a difference in colour between sporelings, whereas diploid spores from the same cystocarp, which share the same genotype, did not exhibit delineation. As *C. crispus* forms dense stands in the intertidal, coalesced holdfasts probably play an important role in population dynamics (see also Plumb 1999). Characterizing the frequency of mixed holdfasts in natural populations will enable a better understanding of the impacts of kin structuring on the mating system and may therefore explain the large F_{IS} values.

Clumped dispersal leads to intergametophytic selfing

Molecular and developmental systems of self-incompatibility exist in plants and animals, such as dioecy or self-incompatibility alleles, to prevent selfing, but are absent in haploid–diploid species (Billiard *et al.* 2012). Dioecy does not prevent intergametophytic selfing which is homologous to the self-fertilization (the fusion of sperm and egg from different gametophytes with both gametophytes originating from the same parental sporophyte). Mating compatibility is determined by the genotype of the haploid nucleus and thus cannot prevent selfing. Haploid–diploid species were reported to be able to undergo both outcrossing and selfing such as fungi (Billiard *et al.* 2012), mosses (Eppley *et al.* 2007), ferns (de Groot *et al.* 2012) and seaweeds (see references hereafter). Nevertheless, some life history traits such as those related to long-distance dispersal of haploid genotypes should result in the prevalence of outcrossing rather than inbreeding. The degree of selfing and/or inbreeding in natural populations of haploid–diploid organisms has, surprisingly, been rarely measured as compared to plants and animals. In fungi, Billiard *et al.* (2012) reported rare heterozygote deficiency from the few studies in which F_{IS} has been measured, such as in the basidiomycetes, which is as expected based on their life history traits. These species disperse primarily as haploid basidiospores just before mating which greatly favours outcrossing. In the red alga *G. gracilis*, single-locus F_{IS} estimates were generally negative, suggesting a slight excess of heterozygotes (Engel *et al.* 2004). In this species, fertilization distance was generally <1 m (Engel *et al.* 1999) but the persistence of the haploid stage, the high density of male and female gametophytes and the occurrence of gene flow probably minimized the probability of mating between sibling gametophytes.

In *C. crispus*, the large F_{IS} values detected were likely due to high levels of inbreeding, specifically intergametophytic selfing. First, the low haploid estimates of null

allele frequency suggested although null alleles may contribute to the large F_{IS} values, they do not solely explain these patterns. The diploid estimates were likely biased due to the assumptions of programs, such as ML-NullFreq, which assume random mating. Alternatively, the high proportion of null alleles detected at some of the highly polymorphic loci may be due to problems scoring alleles or due to allele drop out. Second, the general absence of spatial autocorrelation suggests intergametophytic selfing was the factor underlying large F_{IS} values. In *C. crispus*, the release of tetraspores occurs in chains of gelatinous extrusion (Fredericq *et al.* 1992), which might facilitate clumped recruitment of sibling tetraspores. Significant nonrandom mating may occur between relatives if fertilization distance is short due to sibship structure. Strong positive interactions, such as merging, and kin selection may increase survival, mechanical stability and reduce competition among related individuals. For example, tree clusters of *Nothofagus pumilio*, a dominant Patagonian species, were composed of multiple genets, some of which were full-sibs, and exhibited higher survival from growth in clusters as compared to single-planted seedlings. In assessing the kin-structure of multi-stemmed tress, Till-Bottraud *et al.* (2012) found high levels of inbreeding and clumps composed of multiple genets, some of which were full-sibs. The edge of a forest could be considered a more stressful environment, not unlike the upper limit of a species' distribution within an intertidal environment. Correspondingly, higher levels of intergametophytic selfing were observed in the high shore *C. crispus* stand, where significant kinship coefficients at 25 cm and significant negative slopes over a scale of 1 m indicated fronds located at 25 cm or less were related to one another. Moreover, lower densities were found in the high shore stand which probably increases the selfing rate and thus F_{IS} (Karron *et al.* 1995; Eppley & Pannell 2007). In contrast, no spatial pattern was found in the low shore stand as longer periods of immersion resulting in a greater degree of openness. Concordantly, lower levels of F_{IS} were detected in the low shore stand.

As previously stated, empirical data in plants have revealed an association between dispersal rate and the mating system (see Hamrick & Godt 1996; Duminil *et al.* 2009). Selfing and endogamous mating systems are associated with high levels of genetic differentiation, likely due to both reduction in gene flow and reduced population sizes caused by inbreeding itself. For example, in the kelp *Postelsia palmaeformis*, significant differentiation was detected as little as 5 m apart due to extremely short distance dispersal which resulted in inbreeding (Kusumo *et al.* 2006). In the *C. crispus* stands, high levels of intergametophytic selfing were accompanied by moderate genetic differentiation. Low, but significant F_{ST} values indicated that the high and low shore stands were differentiated, but still linked via on-going gene flow or recent shared ancestry. Further, genetic clustering analyses demonstrated admixture between the populations but also indicated genetic separation between the stands.

The clumping of tetraspores, which disperse over a variety of spatial scales, may also result in large values of F_{IS} and moderate values of F_{ST}. Significant genetic differentiation between high shore plots of gametophytes suggests that at distances of ~50 m, tetraspore dispersal becomes restricted. Thus, at larger spatial scales, levels of genetic differentiation may be higher, not only between gametophytes, but perhaps also between tetrasporophytes. Therefore, although fertilization distance might be short, spore dispersal probably occurs over different spatial scales within the intertidal seascape where spores disperse and recruit in clumps, which homogenizes the genetic structure over a larger scale within a stand up to ~50 m.

Haploid–diploid and intergametophytic selfing

Biphasic life histories probably influence the predominance of intergametophytic selfing as well as its consequences for fitness. The haploid stage in a haploid–diploid life cycle could provide increased efficiency in purging genetic loads. This hypothesis has been used to explain the lack of inbreeding depression detected in some species, such as the sea palm kelp *P. palmaeformis*, in which intergametophytic selfing did not affect individual size or reproduction (Barner *et al.* 2011). However, Raimondi *et al.* (2004) found the costs of intergametophytic selfing in the giant kelp *Macrocystis pyrifera* to be high, where fitness was significantly lowered in selfed progeny as compared to outcrossed progeny. The authors observed the effects of intergametophytic selfing manifested in reproduction in the diploid sporophyte stage (Raimondi *et al.* 2004). The difference between the two studies could be due to generation time where the annual *P. palmaeformis* may have greater selective pressure to ensure persistence via selfing for reproductive assurance, whereas the perennial *M. pyrifera* may have greater selective pressure to prevent selfing due to inbreeding depression (Duminil *et al.* 2009). Alternatively, the huge effective population size in *M. pyrifera* may decrease the chances of effective purging.

Kelp life histories are heteromorphic with a dominant diploid phase. In contrast, some red algal life histories are isomorphic which probably impacts the consequences of selfing. In *C. crispus*, first results do not suggest a strong genetic load, where full-sib male and

female gametophytes produce tetrasporophytes which germinate and grow (Krueger-Hadfield *et al.*, unpublished data). The reproductive viability of the tetrasporophytes produced from full-sib matings would need to be compared to tetrasporophytes produced from nonrelated gametophytes. The persistence and prevalence of intergametophytic selfing in all the *C. crispus* populations studied and the viable spores produced by full-sib mating suggest the effects of inbreeding depression may be low in *C. crispus*. This could be due to genetic purging in the haploid stage (Otto & Marks 1996) or that as the populations are highly inbred, the levels of inbreeding depression are reduced (Crnokrak & Barrett 2002).

As the high shore stand experienced higher levels of intergametophytic selfing, the effects of inbreeding depression should be explored in relation to tidal height as similar patterns are found over the intertidal distribution of this species as found over the latitudinal distribution (e.g. geographic parthenogenesis in *Mastocarpus papillatus*, Fierst *et al.* 2010). Moreover, re-colonization of the North Atlantic following the last glacial maximum may have led to reductions in genetic diversity due to founder events, thereby impacting mating system evolution. Indeed, genetic diversity was lower at higher latitudes than compared to the Iberian Peninsula in *C. crispus* (Provan & Maggs 2012). If analogous patterns as found within an intertidal shore occur, then perhaps populations located at higher latitudes might also display increased levels of intergametophytic selfing associated with historic range changes (see Barringer *et al.* 2012).

The intertidal seascape and genetic structure in Chondrus crispus

Local adaptation and evolutionary divergence among populations may be predicted if gene flow is restricted due to landscape features. There are numerous examples in the literature of landscape barriers to gene flow, such as small changes in altitude which lead to large changes in temperature, humidity and other environmental variables (reviewed in Storfer *et al.* 2010). Giordano *et al.* (2007) demonstrated greater levels of gene flow and genetic diversity at low altitude sites, accompanied by significant genetic differentiation from high altitude sites in the long-toed salamander, *Ambystoma macrodactulym*. In the marine environment, small changes in abiotic parameters associated with depth or tidal elevation have profound impacts on species' distributions, but the scale at which these occur can be much smaller than in landscape genetic studies. In subtidal habitats, thermoclines have been found to reduce gene flow and may lead to local adaptation to temperature and/or depth in different sessile invertebrates (e.g. corals: *Corallium rubrum*, Torrents *et al.* 2008; *Seriatopora hystrix*, Bongaerts *et al.* 2010; the red gorgonian *Paramuricea clavata*, Mokhtar-Jamaï *et al.* 2011). However, although gene flow may be restricted in subtidal environments, the degree of genetic structure is probably higher in intertidal environments. Coleman *et al.* (2011b) reported high connectivity for the subtidal macroalgae *Ecklonia radiata* and *Phyllospora comosa*, whereas the intertidal *Hormosira banksii* was characterized by low connectivity and a strong pattern of isolation by distance. Moreover, Valero *et al.* (2011) reviewed 17 studies of kelps in which intertidal species, whatever their reproductive mode, were more genetically structured than subtidal species, likely due to limited spore and/or gamete dispersal along the shore at low tide. In the intertidal, Engel *et al.* (2004) documented reduced gene flow between high shore and low shore populations in the red alga *G. gracilis*. Even in *C. crispus*, a species continuously distributed throughout the mid-intertidal, significant genetic differentiation occurred within the shore, probably driven by tidal cycles of emersion and immersion. The high shore populations of *C. crispus* at the Port de Bloscon were emerged 55% of the recorded year, whereas the low shore populations were emerged 26% of the time. This difference probably impacts within-shore-level gene flow and the orientation of gene flow between shore levels.

The *C. crispus* zone sampled was ~30 m × 90 m. Yet, the results of this study clearly indicated that the high shore was more isolated as (i) genetic differentiation was evident at distances over 50 m, (ii) inbreeding levels were higher, (iii) there was significant spatial autocorrelation at 25 cm, (iv) a significant decrease in relatedness from 25 cm to 1 m and (v) genetic diversity was lower. Hierarchical sampling of a larger portion of the shore across several different intertidal zones will provide a clearer picture of gene flow. Yet, these results suggest restricted gene flow between shore levels and possibly restricted gene flow higher on the shore. Interestingly, the low shore individuals which exhibit a large proportion of the grey (high shore) cluster were located below a break in the boulder barrier at the Port de Bloscon, forming a channel, perhaps facilitating gene flow from high to low shore through rivulets which move down the shore during low tide or, alternatively, propagules moving from the low shore to high shore on the flowing tide. Engel & Destombe (2002) demonstrated that cystocarp production varied according to tidal height in which high shore pool fertilization happened at low tide, whereas there was no difference between high and low tide fertilization in low shore pools. An analogous study is necessary in *C. crispus* to determine when fertilization and spore release occurs to

understand more about gene flow and what occurs after dispersal at different tidal heights.

To investigate shore-level phenomena within a species, next generation sequencing techniques, such as RAD-seq (reviewed in Davey *et al.* 2011), will enable the detection of loci potentially under selection, providing a clearer picture of gene flow and selection along tidal gradients. At a fine scale within a shore, this study revealed patterns between core and marginal habitats, suggesting local adaptation could be possible between shore levels. These results warrant further investigation of genetic differentiation linked with phenotypic differentiation.

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This article is part of the dissertation research of S.A.K.-H. which focused on the population dynamics of the red seaweed *Chondrus crispus*. She is now a postdoctoral researcher interested in the population dynamics of marine organisms, specifically how dispersal and mating systems affect population structure. D.R. is a researcher interested in evolutionary genetics, specifically the evolution of life cycles. S.M. is a research technician providing support for molecular studies of population structure. M.V. is the director of the BEDIM research group investigating the population genetics and mating systems of marine seaweeds.

Data accessibility

Microsatellite primer sequences were deposited in GenBank (see Table 1 for accession numbers). Microsatellite genotypic data deposited in DRYAD entry doi: 10.5061/dryad.751p3.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Example of the fluctuations in temperature experienced over 1 month (August 2010) in a high and low population of *Chondrus crispus* at the Port de Bloscon, Brittany, France.

Fig. S2 The location of *Chondrus crispus* tetrasporophytes (T), vegetative gametophytes (G), female gametophytes (♀) and male gametophytes (♂) sampled in the (a) high 5 m grid and (b) low 5 m grid at the Port de Bloscon.

Fig. S3 The variation in the mean number of alleles with population size for each of the diploid and haploids subpopulations in the 5 m × 5 m grids and along the transects for n = 5, 10, 15, 20, 25, 30, 50 and 100.

Fig. S4 Spatial auto-correlation plots for the Port de Bloscon 5 m × 5 m grids and transects using Ritland's (1996) kinship coefficient as calculated in the program SPAGEDI.

Table S1 The frequency of null alleles at each locus in each *Chondrus cripsus* stand estimated amongst the haploids and diploids separately in the (a) 5 m grids and (b) transects at the Port de Bloscon.

Table S2 The effect of variation between *Chondrus crispus* populations, ploidies and loci on (a) H_E and b) A_E in the 5 m × 5 m grids and (c) H_E and (d) A_E along the transects at the Port de Bloscon using three-way ANOVA.