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Unravelling the complexity of salt marsh '*Fucus cottonii*' forms (Phaeophyceae, Fucales)

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ABSTRACT

Genetic affiliation, nuclear DNA content, and gamete functioning were examined in small salt marsh *Fucus* from three localities in western Ireland. Individuals with small and dioecious receptacles were found at all localities, but production of germlings was only evident at Locality 1. Here, the *Fucus* vegetation formed a morphological cline from *F. vesiculosus* with bladders in the mid-intertidal to small *Fucus* individuals lacking bladders in the salt marsh of the upper intertidal. Measurements of nuclear DNA content ranged from 1–1.8 pg at this locality, with *F. vesiculosus* individuals in the lower range. At the two other localities, the small salt marsh *Fucus* consisted of distinct morphological entities. Microsatellite analyses revealed that individuals at Locality 2 were derived mainly from *F. vesiculosus*, whereas those from Locality 3 were hybrids between *F. vesiculosus* and *F. spiralis* with greatest affiliation to *F. spiralis*. While the small salt marsh *Fucus* forms from Locality 2 had high nuclear DNA content (c. 4 pg) and were probably octoploids, the small salt marsh *Fucus* from Locality 3 formed two groups: one with high (3.9–4.6 pg) and one with low (1.5–1.9 pg) nuclear DNA content. Nuclear DNA content measured in individuals from Locality 3 varied between 1.1–2.8 pg in *F. vesiculosus* and 2–3.5 pg in *F. spiralis*, and showed a more or less stepwise increase in both species, consistent with polyploidy. We hypothesize that the small salt marsh *Fucus* forms originate from genome size changes in the parental taxa.

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KEYWORDS *Fucus cottonii*; *Fucus spiralis*; *Fucus vesiculosus*; hybridization; nuclear DNA content; polyploidy


Introduction

The brown macroalgal genus *Fucus* is remarkably adaptive and consequently has evolved into several species in the North Atlantic since the migration of ancestral forms from the North Pacific across the Bering Strait (Cánovas *et al.*, 2011). Two main lineages are apparent within the genus, one consisting of *F. distichus* L. and *F. serratus* L. and the other one comprising several species including *F. spiralis* L. and *F. vesiculosus* L., all of which are widespread on shores of the North Atlantic (Cánovas *et al.*, 2011). *Fucus vesiculosus* and *F. spiralis* are closely related and frequently hybridize (Engel *et al.*, 2005) and two new European species within the *F. vesiculosus*–*F. spiralis* lineage have been recognized: *F. radicans* L. Bergström & L. Kautsky from the Baltic Sea (Bergström *et al.*, 2005) and *F. guiryi* G.I. Zardi, K.R. Nicastro, E.S. Serrão & G.A. Pearson, distributed from Ireland and Britain to the Canary Islands (Zardi *et al.*, 2011). *Fucus radicans* has evolved in sympatry with *F. vesiculosus* after the Last Glacial Maximum in Europe (Pereyra *et al.*, 2009), whereas *F. guiryi* (*F. spiralis*-south

in Coyer *et al.*, 2011) is a sister species of *F. spiralis* (Cánovas *et al.*, 2011).

A miniaturized *Fucus* form without holdfasts occurring only in high intertidal salt marshes has been described as *F. cottonii* M.C. Wynne & Magne (Wynne & Magne, 1991). Another small *Fucus* species, *F. chalonii* Feldmann, has similarities with *F. cottonii* but is not associated with salt marsh habitats and only occurs in a few localities on rocky shores in northern Spain (Feldmann, 1941). The small (1–5 cm tall) *F. chalonii* is distinguished from the small salt marsh forms by always being attached and by forming verrucose receptacles.

The small salt marsh form of *F. cottonii*, however, does not represent one genetic entity. Molecular studies have demonstrated that it can be a hybrid between *F. vesiculosus* and *F. spiralis* in the USA and Iceland (Wallace *et al.*, 2004, Coyer *et al.*, 2006), and either a polyploid form of *F. vesiculosus* (Coyer *et al.*, 2006) or derived from *F. spiralis* (Neiva *et al.*, 2012) in Ireland. Miniaturized salt marsh forms have also been

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Fig. 1. Co-occurring morphological forms. Individuals of small salt marsh *Fucus* with narrow thalli adjacent to *F. spiralis* with broader thalli and receptacles (examples shown by arrows) at Locality 3 (Achill Sound) in June 2014.

observed in *F. distichus* from the *F. distichus*–*F. serratus* lineage, suggesting that small salt marsh fucoids represent convergent growth forms (Neiva *et al.*, 2012). However, while growth and morphology of small salt marsh *F. cottonii* was shown to vary with tidal level in a transplant study in a salt marsh locality in Maine, USA (Mathieson *et al.*, 2006), high stress does not fully explain the habit of these small salt marsh fucoids. For example, *Fucus* individuals with normal morphology are often found growing side by side with the miniaturized forms (Fig. 1), and the latter retain their miniaturized morphology when incubated for several weeks in culture with normal salt water (K. Sjøtun, pers. obs.).

A characteristic feature of small salt marsh *F. cottonii* (hereafter abbreviated ssm*F*) is their vegetative reproduction, by which side branches eventually form new, but genetically identical individuals (Cotton, 1912). However, ssm*F* from Ireland occasionally develop receptacles, and the original description of *F. cottonii* (as *F. vesiculosus* var. *muscooides* Cotton) included individuals with small and dioecious receptacles (Cotton, 1912). On the other hand, receptacles have not been observed in ssm*F* in France (Wynne & Magne, 1991), the USA (Mathieson *et al.*, 2006) or in Norway (K. Sjøtun, pers. obs.).

The findings of Coyer *et al.* (2006) and Neiva *et al.* (2012) that the ssm*F* forms can have multiple independent origins suggest some common processes leading to a miniaturized growth form. Coyer *et al.* (2006) showed that ssm*F* could display at least partial genome duplication, suggesting that processes involved in genome size changes can be an underlying cause of miniaturization of *Fucus*. To address this possibility, we collected ssm*F* forms from three sites in western Ireland including Achill Sound,

which is close to the type locality of *F. cottonii*. Microsatellite analysis was combined with analysis of nuclear DNA content (from DAPI analysis of nuclear DNA) and tests of gamete functionality.

Materials and methods

Sampling sites and collections

Small salt marsh forms of *Fucus* were collected from three localities on the western coast of Ireland (Fig. 2) in June 2012 and June 2014. Locality 1 is situated in an enclosed estuary north-west of Galway (Illaunnginga, 53°23'02N, 09°33'50W) and Locality 2 is in the bay outside Clifden (53°29'04N, 10°01'49W). At Locality 3, most individuals were collected on the shore below Achill Sound village (53°55'53.22"N, 09°56'48.44"W), with additional specimens collected at a similar site around 2.5 km further south within Achill Sound (53°54'36.23"N, 09°57'26.87"W).

At Locality 1 (Illaunnginga), the *Fucus* vegetation consisted of *F. vesiculosus* attached to rock in the lower zone, gradually transforming into small salt marsh-like individuals in mud and turf substrata of the middle and upper zones. In the middle zone, *Fucus* individuals possessed small and evesiculate thalli (up to about 10 cm) with rounded receptacles (Fig. 3), whereas in the upper zone, the individuals decreased further in size and all were vegetative. At both Locality 2 (Clifden) and Locality 3 (Achill



Fig. 2. Sample localities in Ireland. *Fucus* spp. were sampled during 2012 and 2014 at Locality 1 (Illaunnginga), Locality 2 (Clifden) and Locality 3 (Achill Sound).



Figs 3–5. Small salt marsh *Fucus* with receptacles from: **Fig. 3.** Locality 1 (Illaunnginga), **Fig. 4.** Locality 2 (Clifden) and **Fig. 5.** Locality 3 (Achill Sound). Scale bar is 1 cm.

Sound), the *Fucus* vegetation consisted of *F. vesiculosus*, *F. spiralis* and *ssmF*, with *ssmF* growing in the upper part of the *Fucus* zone at both localities. Some

Table 1. Sampling specifics. Dates of field sampling, taxa sampled and numbers of individuals per taxon examined or processed are presented for: *Fucus spiralis* (*Fs*), *F. vesiculosus* (*Fv*) and small salt marsh *Fucus* (*ssmF*).

| Collection information | Locality 1 (Illaunnginga) | Locality 2 (Clifden) | Locality 3 (Achill Sound) |
|---|------------------------------------|--|--|
| 14 & 18 June 2012, taxa sampled: | <i>Fv</i> , <i>ssmF</i> | <i>Fv</i> , <i>Fs</i> , <i>ssmF</i> | <i>Fv</i> , <i>Fs</i> , <i>ssmF</i> |
| Individuals of <i>ssmF</i> examined under microscope | 15 | 11 | 17 |
| Individuals of <i>ssmF</i> with receptacles examined | 15 | 11 | 27 |
| Dried for micro- satellite analyses; taxon (no.) | <i>Fv</i> (10) <i>ssmF</i> (15) | <i>Fv</i> (10) <i>Fs</i> (10) <i>ssmF</i> (15) | <i>Fv</i> (20) <i>Fs</i> (23) <i>ssmF</i> (29) |
| DNA extraction; taxon (no.) | <i>Fv</i> (10) <i>ssmF</i> (15) | <i>Fv</i> (8) <i>Fs</i> (8) <i>ssmF</i> (8) | <i>Fv</i> (16) <i>Fs</i> (16) <i>ssmF</i> (16) |
| Individuals success- fully genotyped; taxon (no.) | – | <i>Fv</i> (6) <i>Fs</i> (6) <i>ssmF</i> (5) | <i>Fv</i> (14) <i>Fs</i> (15) <i>ssmF</i> (6) |
| Voucher samples; taxon (no.) | <i>ssmF</i> (10) | <i>ssmF</i> (12) | <i>Fv</i> (1) <i>Fs</i> (1) <i>ssmF</i> (20) |
| 22–23 June 2014, taxa sampled: | <i>Fv</i> , <i>ssmF</i> | <i>ssmF</i> | <i>Fv</i> , <i>Fs</i> , <i>ssmF</i> |
| Fixated for nuclear DNA content analyses; taxon (no.) | <i>Fv</i> (9) <i>ssmF</i> (16) | <i>ssmF</i> (10) | <i>Fv</i> (5) <i>Fs</i> (10) <i>ssmF</i> (12) |
| Nuclear DNA content analyses; taxon (no.) | <i>Fv</i> (3) <i>ssmF</i> (3) | <i>ssmF</i> (3) | <i>Fv</i> (3) <i>Fs</i> (5) <i>ssmF</i> (7) |

ssmF individuals bearing receptacles were found at both Localities 2 and 3 (Figs 4, 5), but less abundantly than at Locality 1. *Fucus spiralis* formed a broad band above *F. vesiculosus* at Locality 3, but was less abundant at Locality 2. Individuals of all taxa were collected haphazardly but normally separated by at least one metre. An overview of sampling dates, collection data and numbers of individuals analysed is given in Table 1. The sampled material was transported back to the laboratory in a cool bag.

Gamete functionality test

Individuals of *ssmF* bearing receptacles were collected at all three localities during June 2012, and receptacles from 11–27 individuals per site were examined, i.e. one receptacle per individual (Table 1). A microscope was used for the examination of *ssmF* individuals and receptacles. Separate receptacles with male and female gametes were found at Localities 1 and 3, and a test of gamete functionality was conducted with material from these localities. For each locality, three receptacles of each sex that appeared to be mature were dried at air temperature for 2.5 hours to initiate gamete release. Thereafter, pairs of male and female receptacles were added to small beakers with sterile unenriched seawater, incubated in a culture chamber (SanyoMLR-351, Sanyo Electric Co., Osaka, Japan) at

10°C with a photon fluence rate of 20–50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (18:6 h light:dark), and examined for released eggs or developing germlings after 1, 4 and 6 days.

Microsatellite analyses

In order to determine the genetic origins of *ssmF*, samples of *F. vesiculosus* (all localities), *F. spiralis* (Localities 2 and 3) and *ssmF* (all localities) were collected for microsatellite analyses in June 2012. After returning to the laboratory, one clean (no epiphytes) branch tip per individual was excised and dried in silica gel crystals. Tissue from 10–29 individuals per taxon per locality was collected (Table 1). Individuals other than those examined for gamete functionality were selected for microsatellite analyses to ensure extraction of high quality DNA. Vouchers of 10–20 *ssmF* were preserved from each locality (photos of pressed voucher specimens are added as Supplementary figs S1, S2, S3–1, S3–2). DNA was extracted from 8–16 individuals per taxon per locality using a modification of the CTAB method (Coyer *et al.*, 2009) and purified with Zymo One-Step-96 PCRTM Inhibitor Removal Kit (Zymo Research) following the manufacturer's protocol. 8–16 individuals of each taxon per locality (Table 1) were genotyped using four microsatellite loci (L20, L38, L58 and L94) and the protocols by Engel *et al.* (2003). Due to methodological problems, no individuals from Locality 1 were successfully amplified, thus this locality was not included in the microsatellite analyses. Genotypes were visualized on an ABI 3730 gene analyser (Applied Biosystems) and analysed using GENOTYPER (Applied Biosystems) software.

Genetic diversity measured as Nei's non-biased H_{exp} (Nei, 1978) and estimators of F_{IS} and F_{ST} (Wright, 1969) (as f and θ ; Weir & Cockerham, 1984) were calculated using GENETIX 4.02 (Belkhir *et al.*, 2001). Standardized allelic richness was determined for each population with GENCLONE 2.0 (<http://wwz.ifremer.fr/clonix/Logiciels/GenClone-2.0>).

Clustering of microsatellite genotypes used STRUCTURE (Pritchard *et al.*, 2000) through the Lifeportal computing service at the University of Oslo. STRUCTURE uses a Bayesian algorithm to identify clusters or groups of genetically homogeneous individuals (K), and has been used to study hybridization in *Fucus* (e.g. Engel *et al.*, 2005, Coyer *et al.*, 2006). Analyses were run with $K=1-6$, and each run was replicated 10 times to ensure proper convergence of the MCMC with the parameters: ancestry model = admixture (to account for recent divergence and shared ancestral polymorphisms); frequency model = independent; burn-in = 1 000 000; MCMC length = 2 000 000 after burn-in. Since *ssmF*

show vegetative reproduction and potentially form clones and *F. spiralis* has a high degree of selfing (Engel *et al.*, 2005), clustering of the microsatellite genotypes was also examined with INSTRUCT (Gao *et al.*, 2007), which eliminates the assumption of Hardy–Weinberg equilibrium within clusters and calculates expected genotype frequencies on the basis of inbreeding or selfing rates. In both cases the most likely K value was inferred from the rate of change in estimated Ln probability of data between successive K values (ΔK) (Evanno *et al.*, 2005), using STRUCTURE HARVESTER web version 0.6.94 (Earl & vonHoldt, 2012) and CLUMPAK beta version (Kopelman *et al.*, 2015). The results were visualized with DISTRUCT 1.1 (Rosenberg, 2004). The genetic affinities of *ssmF* from Localities 2 and 3 were further analysed with principal component analysis (PCA) in R 3.2.5 using the R package ADEGENET (Jombart, 2008). Genotype spreading was very wide in *F. vesiculosus* from Locality 3, and two extreme outliers were removed before the final PCA. Multivariate statistical methods such as PCA are free from assumptions of underlying population genetic models and represent an independent method of measuring population differentiation (Jombart *et al.*, 2010).

Nuclear DNA content analyses

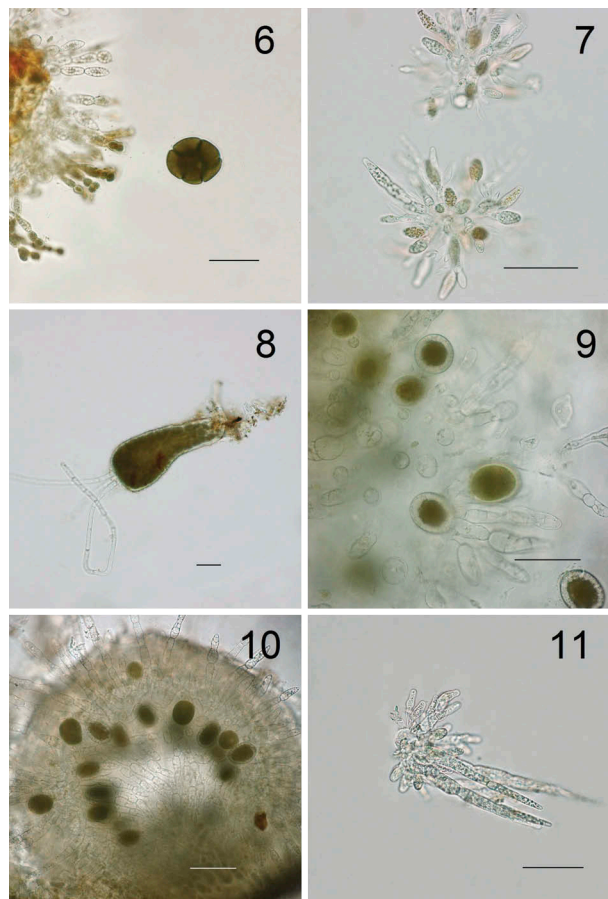
To detect variation in genome size and possible polyploidy in the *ssmF* individuals, nuclear DNA content was estimated with microspectrofluorometry analyses of DAPI stained tissue. Individuals of *ssmF* collected at all three localities in June 2014 were included, but *F. vesiculosus* and *F. spiralis* were only collected from Localities 1 and 3, and Locality 3, respectively (Table 1), since we initially did not expect a large variation in nuclear DNA content of these species on a small spatial scale (Gómez Garreta *et al.*, 2010). A small tissue section was excised from each individual and fixed in Carnoy's solution (3:1 100% ethanol:glacial acetic acid, mixed just before use) for 24 h, then stored in 70% ethanol at 4°C. Fixation in Carnoy's solution prevents staining inhibition due to phlorotannins. Nuclear DNA content was estimated by image analysis using METAMORPH software (Molecular Devices, Toronto, Ontario, Canada) and comparing the If (Intensity of fluorescence) of *Fucus* nuclei with those of chicken erythrocytes, which have a DNA content of 2.4 pg (Clowes *et al.*, 1983). Analyses of nuclear DNA content were carried out using an AxioCam HRm Zeiss digital camera on an AxioPlan Zeiss fluorescence microscope (see Gómez Garreta *et al.*, 2010). Estimates of nuclear contents were obtained from 3–7 *ssmF* from each locality (Table 1). Additionally, nuclear contents were estimated for three *F. vesiculosus* individuals from Localities 1 and 3, and five *F. spiralis* individuals

from Locality 3. Mean nuclear DNA contents per individual were based on 68–247 nuclei (Table 4).

Results

Gamete functionality test

Since *Fucus* species can be dioecious (male antheridia and female oogonia on separate individuals, e.g. *F. vesiculosus*) or hermaphroditic (male antheridia and female oogonia within a single receptacle, e.g. *F. spiralis*), *ssmF* receptacles were examined in order to sex the individuals. Only female receptacles ($n = 11$) were found at Locality 2 (Fig. 9), while eight female and six male receptacles were found at Locality 1 (Figs 6, 7), and 11 female and six male receptacles at Locality 3 (Figs 10, 11). The male receptacles from Locality 3 had relatively few conceptacles. No hermaphroditic receptacles of the *ssmF* individuals were found, except for four of 25 receptacles from Locality 3, which had oogonia and structures that may have been immature antheridia. A few



Figs 6–11. Conceptacle structure and germling growth. **Fig. 6.** Mature oogonium containing egg cells ready to be released; **Figs 7–8.** Antheridia (Fig. 7) and germlings (Fig. 8) after 6 days in culture from Locality 1 (Illaunnginga) (possible damage to the attachment rhizoids due to transfer to microscope slide). **Fig. 9.** Immature oogonia from Locality 2 (Clifden). **Fig. 10.** Immature oogonia. **Fig. 11.** Antheridia both from Locality 3 (Achill Sound). Scale bar is 50 μm .

sterile receptacles were found at both Localities 2 and 3. Receptacles of *ssmF* from Locality 1 were normal in appearance with viable and functioning gametes, and after 6 days numerous normal-looking germlings were observed in the incubated beakers (Fig. 8). The *ssmF* individuals from Locality 1 differed further from those of the other localities by having a more conspicuous mid-rib (Fig. 3). Only a few oogonia with division lines were observed in receptacles from Localities 2 and 3, and oogonia from these localities frequently were abnormal with cell contents concentrated in the middle (Figs 9, 10). None of the incubations from Locality 3 produced germlings.

Microsatellite analyses

The microsatellite analyses gave results for 5–6 individuals of each taxon from Locality 2, and for 6–15 individuals per taxon from Locality 3. Significant F_{IS} values were found for all three taxa from Locality 3 and for *F. spiralis* from Locality 2, which suggests more inbreeding in these populations than in the others (Table 2). A relatively high expected heterozygosity ($H_{exp} = 0.813$) was found for *F. vesiculosus* at Locality 3 (Table 2), probably reflecting the larger sampling area. Pairwise F_{ST} comparisons revealed an overall greater genetic distance among populations at Locality 3 than among those at Locality 2, except for the comparison between *F. spiralis* and *F. vesiculosus*, which showed a lower F_{ST} value at Locality 3 than at Locality 2 (Table 3). *Fucus spiralis* showed the highest inbreeding coefficients (F_{IS}) at Localities 2 and 3. At Locality 3 also *ssmF* and *F. vesiculosus* had significant inbreeding coefficients (Table 2). The microsatellite analyses did not reveal clones of *ssmF*. Allelic richness

Table 2. Calculated F_{IS} , H_{exp} and allelic richness (\bar{A}) for n individuals from populations of *Fucus spiralis* (*Fs*), *F. vesiculosus* (*Fv*) and small salt marsh *Fucus* (*ssmF*) from Locality 2 (Clifden) and Locality 3 (Achill Sound).

| Locality-taxon | n | F_{IS} | H_{exp} | \bar{A} |
|----------------|-----|----------|-----------|-----------|
| 2- <i>Fs</i> | 6 | 0.590* | 0.327 | 1.400 |
| 2- <i>ssmF</i> | 5 | 0.271 | 0.513 | 2.800 |
| 2- <i>Fv</i> | 6 | 0.102 | 0.570 | 2.726 |
| 3- <i>Fs</i> | 15 | 0.618* | 0.295 | 1.472 |
| 3- <i>ssmF</i> | 6 | 0.510* | 0.374 | 2.600 |
| 3- <i>Fv</i> | 14 | 0.504* | 0.813 | 4.266 |

Significant F_{IS} values are marked with an asterisk. Allelic richness (\bar{A}) is standardized to $n = 5$.

Table 3. Pairwise F_{ST} values comparing populations of *Fucus spiralis* (*Fs*), *F. vesiculosus* (*Fv*) and small salt marsh *Fucus* (*ssmF*) from Locality 2 (Clifden) and Locality 3 (Achill Sound).

| Locality-taxon | 2- <i>ssmF</i> | 2- <i>Fv</i> | 3- <i>Fs</i> | 3- <i>ssmF</i> | 3- <i>Fv</i> |
|----------------|----------------|--------------|--------------|----------------|--------------|
| 2- <i>Fs</i> | 0.408 | 0.447 | -0.059 | 0.469 | 0.238 |
| 2- <i>ssmF</i> | | 0.073 | 0.492 | 0.349 | 0.064 |
| 2- <i>Fv</i> | | | 0.526 | 0.464 | 0.078 |
| 3- <i>Fs</i> | | | | 0.497 | 0.327 |
| 3- <i>ssmF</i> | | | | | 0.182 |

ness for *F. vesiculosus* was higher than for *ssmF* and *F. spiralis* at Locality 3, while at Locality 2 *F. vesiculosus* and *ssmF* had similar and higher allelic richness than *F. spiralis* (Table 2). Information about allele proportions is included in Supplementary table S4.

According to the delta K plots from the STRUCTURE and INSTRUCT analyses, the most likely distribution of the microsatellite genotypes at Localities 2 and 3 (not available for Locality 1) was $K = 2$, followed by $K = 3$. The two clusters obtained by $K = 2$ corresponded largely to the individuals sampled as *F. spiralis* and *F. vesiculosus*. Genotyped *ssmF* individuals from Locality 2 showed highest similarity to *F. vesiculosus* as shown by both STRUCTURE and INSTRUCT (Fig. 12). Genotyped *ssmF* individuals from Locality 3 showed admixture between *F. vesiculosus* and *F. spiralis* according to the STRUCTURE results, but were similar to *F. spiralis* according to the INSTRUCT results (Fig. 12).

With $K = 3$, the STRUCTURE and INSTRUCT analyses showed identical patterns (only the result of the INSTRUCT analysis is shown in Fig. 12). In this case, the *ssmF* from Locality 3 formed a separate

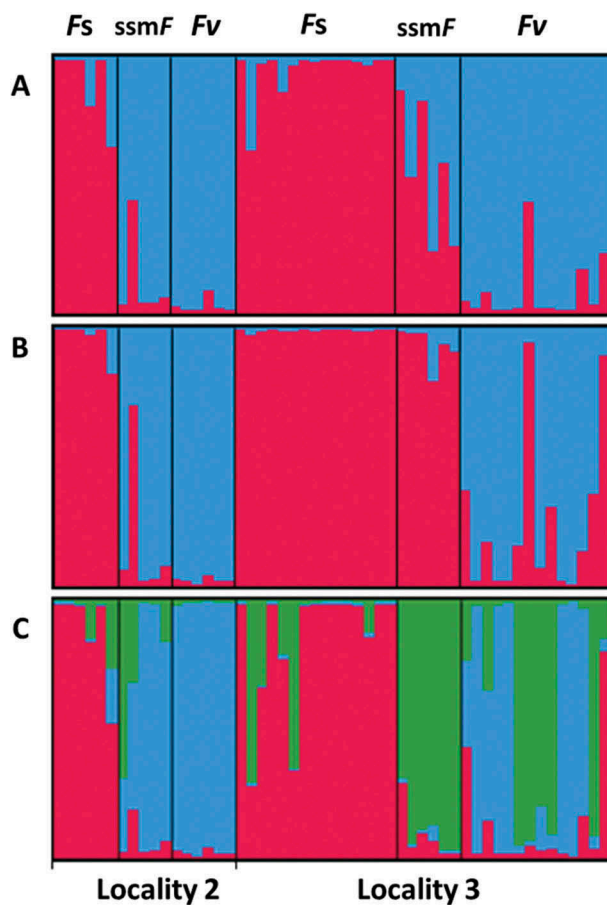


Fig. 12. Results from STRUCTURE (A) and INSTRUCT (B) analyses with $K = 2$; and from INSTRUCT analysis with $K = 3$ (C). Localities 2 and 3 consist of individuals sampled as *Fucus spiralis* (Fs), *F. vesiculosus* (Fv) and small salt marsh *Fucus* (*ssmF*). Each individual is represented by a bar and colours represent the proportional assignment to the STRUCTURE/INSTRUCT groups.

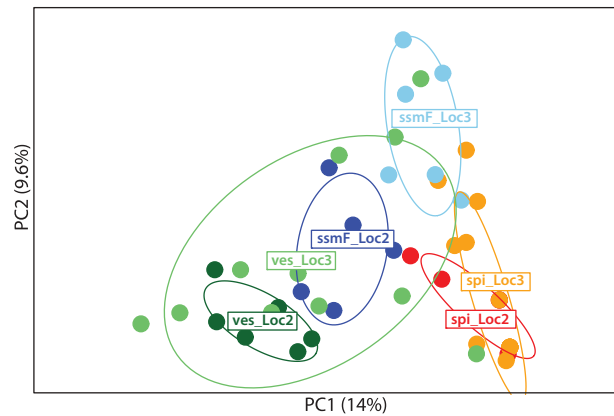


Fig. 13. The two first principal components (PC) of a Principal Component Analysis showing genetic affiliation of haplotype composition of *ssmF* (blue), *F. vesiculosus* (ves) (green) and *F. spiralis* (spi) (orange-red) from Locality 2 (Loc2) and Locality 3 (Loc3). The circles represent 95% inertia ellipses for the populations, which characterizes the dispersion of each population around its centre of gravity. Percentages along axes indicate the proportion of overall variability explained by the principal components.

group, but shared this haplotype combination with some *F. spiralis* and *F. vesiculosus* individuals from the same locality. At Locality 2 the analyses indicated some admixture with *F. spiralis* in some of the *ssmF* individuals.

The results of the PCA analysis largely confirmed the results from the STRUCTURE and INSTRUCT analyses (Fig. 13), except that the *ssmF* individuals clustered in a more intermediate position relative to *F. spiralis* and *F. vesiculosus* at both localities. The high genetic diversity of *F. vesiculosus* from Locality 3 was also evident in the PCA, with these individuals spread throughout the plot, overlapping totally with individuals of *F. vesiculosus* and *ssmF* from Locality 2, and partly with *ssmF* from Locality 3.

Nuclear DNA content analyses

Nuclear DNA content was reported as C-value units (Table 4), with C representing the estimated weight (pg) of the nuclear DNA content of non-replicated gametes. Very few direct C values (from sperm) were obtained from the examined individuals, and none from the receptacle-bearing *ssmF* (Table 4). Members of *Fucus* are characterized by a diplontic life cycle with diploid 2C nuclei in non-replicated vegetative cells (G1-phase) and 4C in replicated cells (G2-phase) (Gómez Garreta *et al.*, 2010). Most recordings of nuclear DNA content were 2C- or 4C-values (Table 4). The 2C-values of three *ssmF* individuals from Locality 1 ranged from 1 to 1.8 pg, whereas the values of the three individuals from Locality 2 ranged from 4 to 4.3 pg (Fig. 14). The *ssmF* individuals from Locality 3 formed two groups, with three individuals

Table 4. Overview of results from estimated nuclear DNA content from Localities (Loc.) 1 (Illaunnginga), 2 (Clifden) and 3 (Achill sound) of small salt marsh *Fucus* (ssmF), *F. vesiculosus* (Fv) and *F. spiralis* (Fs).

| Sample-id | Loc. | Nuclei (n) | 2C (SD) | 4C (SD) | C (SD) | Receptacles |
|-----------|------|------------|----------|----------|----------|-------------|
| ssmF-1 | 2 | 201 | 4(0.3) | 8.4(0.7) | – | No |
| ssmF-2 | 2 | 184 | 4.3(0.8) | 8.2(0.2) | – | Yes |
| ssmF-3 | 2 | 190 | 4(0.3) | 8.4(0.7) | – | No |
| ssmF-11 | 1 | 172 | 1.6(0.3) | 3.3(0.5) | – | No |
| ssmF-19 | 1 | 208 | 1(0.1) | 3.1(0.2) | – | Yes |
| ssmF-20 | 1 | 164 | 1.8(0.4) | 4.3(0.2) | – | Yes |
| Fv-27 | 1 | 231 | 1.3(0.3) | 2.7(0.3) | – | Yes |
| Fv-28 | 1 | 76 | 1(0.3) | 2.3(0.5) | – | Yes |
| Fv-30 | 1 | 167 | 1(0.3) | – | 0.7(0.2) | Yes |
| ssmF-36 | 3 | 87 | 1.8(0.5) | – | – | No |
| ssmF-37 | 3 | 153 | 1.5(0.2) | 3.4(0.4) | – | No |
| ssmF-38 | 3 | 69 | 4.1(0.8) | 7.3(0.5) | – | Yes |
| ssmF-39 | 3 | 71 | 3.9(0.3) | 6.4(0.1) | – | Yes |
| ssmF-40 | 3 | 187 | 4.6(0.6) | 7.9(0.6) | – | Yes |
| ssmF-41 | 3 | 173 | 1.9(0.6) | 4.2(0.5) | – | Yes |
| ssmF-42 | 3 | 154 | 4(0.3) | 7.7(0.4) | – | No |
| Fs-48a | 3 | 247 | 3.5(0.3) | 5.4(0.6) | – | No |
| Fs-48b | 3 | 151 | 2(0.4) | 3.8(0.4) | 0.9(0.2) | Yes |
| Fs-50 | 3 | 104 | 3.3(0.4) | 5.8(0.4) | – | Yes |
| Fs-51 | 3 | 68 | 3.1(1.0) | 5.8(0.8) | – | Yes |
| Fs-52 | 3 | 144 | 3(0.3) | 4.8(0.2) | – | No |
| Fv-57 | 3 | 123 | 1.8(0.3) | 4.4(0.3) | – | Yes |
| Fv-58 | 3 | 83 | 2.8(0.5) | 4.2(0.2) | – | Yes |
| Fv-60 | 3 | 136 | 1.1(0.3) | 2.2(0.3) | 0.6(0.1) | Yes |

The results are given in pg (\pm SD) for 2C, 4C and for C (sperm) when possible. Nuclei (n) = number of nuclei examined per sample. Presence or absence of receptacles in the analysed individuals is indicated.

in the lower (1.5–1.9 pg) and four individuals in the upper (3.9–4.6 pg) range (Fig. 14). The three *F. vesiculosus* individuals from Locality 1 had an overall lower but overlapping range of nuclear DNA content with the ssmF individuals from the same locality (Fig. 14). At Locality 3, the three *F. vesiculosus* individuals showed a variable nuclear DNA content, ranging from 1.1 to 2.8 pg. The *F. spiralis* individuals

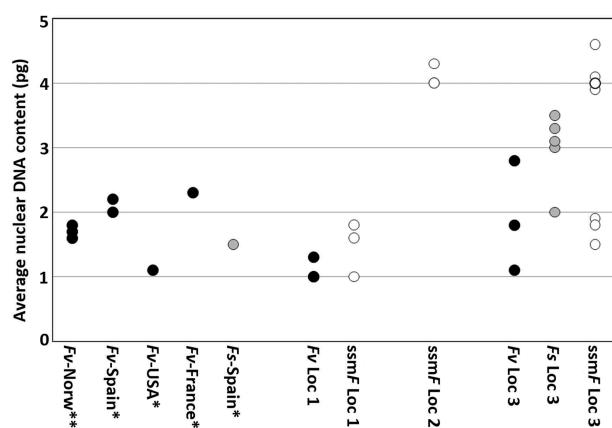


Fig. 14. Average nuclear DNA content (2C; pg) in *Fucus vesiculosus* (Fv, black circles), *F. spiralis* (Fs, grey circles) and small salt marsh *Fucus* (ssmF, white circles). Data from Illaunnginga (Locality 1), Clifden (Locality 2) and Achill Sound (Locality 3) are shown, together with unpublished data from Norway (**) and earlier published data (*) from Spain (Gómez Garreta *et al.*, 2010), USA (Kapraun, 2005) and France (Phillips *et al.*, 2011, recalculation from Peters *et al.*, 2004).

from Locality 3 had 2C estimates of 2–3.5 pg (with four out of the five estimates between 3 and 3.5 pg). Unfortunately, we have no analyses of nuclear DNA content for *F. vesiculosus* and *F. spiralis* from Locality 2.

Discussion

The ssmF showed surprisingly high variation between the three localities in western Ireland with respect to reproduction mode, genetic affiliation and nuclear DNA content. Previous studies have indicated that ssmF can originate from different *Fucus* taxa, even between localities in Ireland (Coyer *et al.*, 2006; Neiva *et al.*, 2012). This is supported by our microsatellite results, which showed that ssmF from Locality 2 were derived mainly from *F. vesiculosus*, whereas the ssmF individuals from Locality 3 revealed a higher degree of hybridization or introgression, but with greatest affiliation to *F. spiralis*. The fact that ssmF had a significant inbreeding coefficient at Locality 3 but not at Locality 2 also suggests a connection of ssmF to *F. spiralis* at Locality 3, since a high degree of inbreeding is found in *F. spiralis*. At Locality 3 the sampling was done at two sites about 2.5 km apart, but the microsatellite clustering did not reveal genetic separation between the sites.

Only ssmF from Locality 1 displayed successful sexual reproduction as shown by a test of gamete functionality. Unfortunately, no individuals from Locality 1 were included in the microsatellite analyses (due to unsuccessful amplification). However, since the *Fucus* vegetation formed a cline from *F. vesiculosus*-like individuals in the lower intertidal to ssmF-like and dioecious individuals in the upper intertidal, and no *F. spiralis* were observed at or in the vicinity of Locality 1, we assume that ssmF at Locality 1 were derived from *F. vesiculosus*. Both *F. vesiculosus* and ssmF from Locality 1 had mature receptacles during June, and are likely to be interfertile.

Coyer *et al.* (2006) examined ssmF from one locality in Ireland and showed that ssmF were polyploids derived from *F. vesiculosus*. To investigate this further, we estimated the nuclear DNA contents of ssmF, *F. vesiculosus* and *F. spiralis*. Generally, we found both much higher estimates of, and variability in, nuclear DNA content than has previously been measured for these *Fucus* taxa, even within a single locality (Fig. 14). Previously published estimates of nuclear DNA content (2C) of *F. vesiculosus* from different geographical areas (Norway, Spain, USA and France; Fig. 14) varied between 1 and 2.3 pg (Kapraun, 2005; Gómez Garreta *et al.*, 2010; Phillips *et al.*, 2011). We obtained estimates between 1 and 2.8 pg for this taxon from two localities less than 100 km apart. The highest nuclear DNA content (up to 4.6 pg) was found in a group of ssmF individuals from Locality 3. Variable genome

sizes have been reported for some brown algae (Kapraun, 2005; Gómez Garreta *et al.*, 2010; Phillips *et al.*, 2011), but little is known about the mechanisms causing such variation.

Variation in genome size has long been studied in land plants, and several mechanisms have been demonstrated (Šmarda & Bureš, 2010; Leitch & Leitch, 2013). Even though it is likely that processes influencing genome size are different in land plants compared with brown algae, which are evolutionarily distant from Archaeplastida (Yoon *et al.*, 2004), there are probably also similarities. For example, a commonly occurring process in land plants is polyploidy, a rapid and dramatic doubling of the genome size sometimes from one generation to the next (Bennetzen *et al.*, 2005), which can, if repeated, result in a range of ploidy levels. Newly evolved polyploids are expected to increase the genome size in direct proportion with ploidy. However, many polyploids also show a reduction over time in DNA content relative to diploids as a result of genome downsizing through various processes (Leitch & Bennett, 2004; Leitch & Leitch, 2013).

Autopolyploids arise within or among populations of a single species by the doubling of homologous chromosome sets. Allopolyploids, on the other hand, combine two or more genomes from different species as a result of interspecific hybridization. In nature, both autopolyploids and allopolyploids often result from the formation of unreduced gametes (Tayalé & Parisod, 2013; De Strome & Mason, 2014) and usually through a triploid intermediate formed by the union of unreduced and reduced gametes. The triploid may in turn produce tetraploids either through selfing, or by crossing with other triploids or diploids. Triploids may produce viable gametes of varying ploidy ($n = x, 2x, 3x$), leading to individuals with variable genome sizes (Mason & Pires, 2015). Diploids and tetraploids in a population may further cross and form triploids, which in turn may produce viable gametes (Birchler, 2012). As more and more population-scale studies on genome size variation are published, it is clear that odd-numbered ploidies exist within species and populations, suggesting that successful intercytotype crossings may be more widespread than previously thought (Husband *et al.*, 2013).

Production of unreduced gametes in land plants occurs more frequently under environmental stress such as low and fluctuating temperatures, water stress and disease (Tayalé & Parisod, 2013; De Strome & Mason, 2014). The estuarine conditions of the localities in the present study provide similar stressful habitats with regard to salinity and temperature variation in the intertidal, which consequently may favour formation of unreduced gametes and further facilitate polyploidization.

Polyploidization, followed by repeated crossings between individuals with different ploidy levels, may explain the variable genome sizes observed in *Fucus* at Locality 3. The lowest 2C value found in *F. vesiculosus* is 1 pg (Kapraun, 2005; this study). Under the assumption that this value represents a diploid genome size, the more or less stepwise increase in genome size suggests that tetraploids and hexaploids were found at Locality 3. If the diploid genome size of *F. spiralis* is *c.* 1.5 pg (as reported from Spain, Fig. 14), most of the *F. spiralis* from Locality 3 could be considered tetraploids with genome sizes around 3 pg. However, with the small number of analysed individuals and the relatively high variation in nuclear DNA estimates, we cannot exclude the possibility that a smaller diploid genome size exists for *F. spiralis* at Locality 3 (e.g. 1 pg). In this case the individual at Locality 3 with a genome size of *c.* 2 pg may be a tetraploid, and those with higher ploidy could be hexaploids.

Assuming polyploid origins, the *ssmF* individuals at Locality 2 with large genome sizes (*c.* 4 pg) can be considered octoploids that have originated mainly from *F. vesiculosus*. The *ssmF* individuals at Locality 3, however, can be separated into two groups, one with genome sizes ≥ 4 pg (which can be interpreted as hexa- or octoploid), and the other with genome sizes of 1.5–2 pg. No sexual recruitment was observed for *ssmF* from Locality 3, suggesting that both groups originated from the reproducing *Fucus* populations at the site. Microsatellite analyses showed that *ssmF* from Locality 3 most likely were hybrids and/or introgressed *F. spiralis*. *Fucus vesiculosus* and *F. spiralis* are closely related and may easily hybridize, but a ‘normal’ F_1 hybrid has an intermediate morphology between the two species (Burrows & Lodge, 1951). We propose that different ploidies and inter- or intraploidy crossings between individuals of *F. vesiculosus* and *F. spiralis* may be responsible for the varying ploidy, deviating morphology, and loss of function among *ssmF* from Localities 2 and 3.

The *ssmF* at Locality 1 seem to represent a special case, since they had functional gametes and sexual reproduction. They also deviated morphologically from the *ssmF* at Localities 2 and 3 by having a more conspicuous mid-rib. Compared to estimates from the other localities, the genome size estimates from Locality 1 were at the lower end of the range. However, *ssmF* here also had the highest genome sizes, though overlapping with those of *F. vesiculosus*. The processes leading to variable genome sizes at Locality 1 are unknown, but are probably due to processes other than polyploidization (e.g. Piegu *et al.*, 2006).

It is not clear what causes the miniaturization of *ssmF* that was observed at all three localities, but it seems to be unrelated to genetic affiliation or genome size *per se* (see also Wallace *et al.*, 2004; Coyer *et al.*, 2006; Neiva *et al.*, 2012). We hypothesize that occurrence of *ssmF* is connected to processes causing variable genome sizes in *Fucus* populations, and that formation of *ssmF* occurs most frequently at localities where one or more members of *Fucus* have an unstable genome. Our hypothesis may explain why *ssmF* are not present in all salt marshes with apparently suitable habitat. For example, Sheehy Skeffington & Curtis (2000) found that the distribution of *ssmF* was limited to the west coast of Ireland, and connected to high precipitation and low salinity in the local salt marshes. A stressful environment may cause genome size instability and variation in plants in various ways (e.g. Mirouze & Paszkowski, 2011; De Strome & Mason, 2014), and estuaries with very low or strongly variable salinity may in a similar way represent stressful biotopes for fucoids, resulting in genome size variations.

Furthermore, in plants it has been demonstrated that high variation in genome size, as a result of polyploidization and subsequent genome downsizing, or activation of epigenetics and transposable elements, can influence gene functioning and expression, through chromosome rearrangements and epigenetic mobilizations (Song & Chen, 2015). Interestingly, a recent study of speciation in angiosperms showed that high rates of phenotypic changes and speciation are positively and strongly correlated with genome size changes, but not with absolute genome size (Puttick *et al.*, 2015). Processes that change genome sizes, therefore, may influence both growth and functioning of organisms. Here, variation in genome size could have resulted in malfunction of genes necessary for normal development of antheridia (male gametangia) in *ssmF* from Locality 2.

Our study shows that members of the genus *Fucus* display a surprisingly large variation in genome size, even within populations. Although processes regulating speciation in angiosperms and brown algae may be quite different, it is possible that the high genome size variation observed in *Fucus* may partially explain the observed high capacity for morphological plasticity. While we cannot determine the underlying processes causing the patterns reported in the present study, our results clearly show that these processes need to be investigated further in *Fucus*. The genus, therefore, may be an excellent model for testing the connection between variable genome sizes, population adaptation to different environmental conditions, and speciation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Supplementary information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at <http://dx.doi.org/10.1080/09670262.2017.1309688>

Supplementary fig. S1. Voucher specimens (6 of 10 pressed) of *ssmF* from Locality 1 Illaunnginga, collected 14 June 2012.

Supplementary fig. S2. Voucher specimens of *ssmF* from Locality 2 (Clifden), collected 14 June 2012.

Supplementary fig. S3–1. Voucher specimens of *ssmF* from Locality 3 (Achill Sound, by Achill Sound village), collected 18 June 2012.

Supplementary fig. S3–2. Voucher specimens of *ssmF* from Locality 3 (Achill Sound, about 2.5 km south of Achill Sound village), collected 18 June 2012.

Supplementary table S4. Proportions of alleles of the four microsatellite loci, L20, L38, L58 and L94.

Author contributions

K. Sjøtun: original concept, field and laboratory work, drafting and editing manuscript; S. Heesch: field work, interpretation and editing manuscript; J. Rull Lluçh, R. Martin Martin, A Gomez Garreta: analysis of DNA content and interpreting of results; A.K. Brysting: interpreting results and editing manuscript; J.A. Coyer: microsatellite analysis, interpreting the results and editing the manuscript.

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