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Development and characterization of 35 single nucleotide polymorphism markers for the brown alga *Fucus vesiculosus*

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We characterized 35 single nucleotide polymorphism (SNP) markers for the brown alga Fucus vesiculosus. Based on existing *Fucus* Expressed Sequence Tag libraries for heat and desiccation-stressed tissue, SNPs were developed and confirmed by re-sequencing cDNA from a diverse panel of individuals. SNP loci were genotyped using the SEQUENOM® single base extension iPLEXTM system for multiplex assays on the MassARRAY® platform, which uses matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to discriminate allele-specific products. The SNP markers showed a wide range of variability among 16 populations from the south-west of the UK, northern Portugal and Morocco. The analysis of the information provided by these markers will be useful for studying population structure, historical demography and phylogeography of *F. vesiculosus*. They can also be used for the identification of genes and/or linked genomic regions potentially subject to selection in response to abiotic stressors like temperature extremes and desiccation intensity that vary across habitats and geographical range.

Key words: expressed sequence tag, fucoid algae, *Fucus vesiculosus*, MassARRAY®, selection, Phaeophyceae, phylogeography, single nucleotide polymorphism, SNP genetic marker

Introduction

Fucus vesiculosus is a member of the brown algal family Fucaceae, which are prominent and widespread ecosystem-structuring components of cold to temperate intertidal communities throughout the North Atlantic Ocean. Ecotypic divergence (e.g. in estuarine and low salinity habitats; Serrão *et al.*, 1996; Pearson *et al.*, 2000; Coyer *et al.*, 2006; Lago-Leston *et al.*, 2010), spatial isolation and restricted gene flow (Engel *et al.*, 2005; Tatarenkov *et al.*, 2005; Perrin *et al.*, 2007; Muhlin & Brawley, 2009), genetically based differentiation in stress tolerance (Pearson *et al.*, 2006, 2009; Zardi *et al.*, 2011), ongoing speciation processes (Tatarenkov *et al.*, 2005; Pereyra *et al.*, 2009) and hybridization (Billard *et al.*, 2005; Engel *et al.*, 2005; Moalic *et al.*, 2011), as well as palaeohistorical shifts in geographical range with glacial cycles (Muhlin & Brawley, 2009; Coyer *et al.*, 2011*a*) all contribute to population differentiation in *F. vesiculosus* at different spatial and temporal scales. The complexity of the interactions in the evolution of this and other fucoid species, involving historical demography (e.g. Hoarau *et al.*, 2007), mating system (e.g. Perrin *et al.*, 2007), selection along steep gradients of abiotic stress (e.g. Coyer *et al.*, 2011*b*; Zardi *et al.*, 2011), hybridization and introgression (e.g. Neiva *et al.*, 2010), and speciation (e.g. Pereyra *et al.*, 2009), requires the design of new genetic markers that allow us to better describe population differentiation processes.

The application of genomic techniques in ecological and evolutionary research on non-model organisms like brown algae is likely to become more widespread as data become available (Cock *et al.*, 2010; Pearson *et al.*, 2010). Molecular marker technology has developed rapidly over the last decade and single nucleotide polymorphisms (SNPs) have now became an important class of marker in modern phylogeography and evolutionary ecology (Garvin *et al.*, 2010). While SNPs are less polymorphic than some alternatives such as microsatellites, they are more abundant throughout the genome and, due to their low mutation rates compared with microsatellites, are also more evolutionarily stable (i.e. less prone

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to recurrent substitutions: Picoult-Newberg *et al.*, 1999; Brumfield *et al.*, 2003). They constitute a source of neutral loci that can be used to improve parameter estimates concerning population demography and structure (Morin *et al.*, 2009). SNP markers, particularly when designed from ESTs (Picoult-Newberg *et al.*, 1999), can also yield loci that have experienced different selective regimes, providing resolution for studies of adaptive evolution (reviewed by Garvin *et al.*, 2010). In these particular cases, the information can be useful for the identification of mutations that are involved in local adaptation (Namroud *et al.*, 2008). The characteristics of SNPs make them excellent markers for understanding genome evolution (Syvanen, 2001), while detection, assay design and genotyping can be automated for high throughput (Morin *et al.*, 2004).

Materials and methods

Candidate gene loci were selected from EST libraries developed from two species of *Fucus*, exposed to either desiccation/rehydration (*F. serratus* and *F. vesiculosus*) or heat shock/recovery (*F. serratus*; Pearson *et al.*, 2010). Candidate unigenes were selected from over 12 000 ESTs contained in these libraries. To identify and confirm SNPs, a resequencing panel specifically for *F. vesiculosus* was designed using three to five individuals from four populations, selected in order to encompass population differentiation at different spatial and environmental scales: Tagus Estuary, Portugal (38° 45′ 29.72″N, 8° 57′ 28.16″W); Viana do Castelo, Portugal (41° 41′ 55.70″N, 8° 51′ 18.11″W); Roscoff, France (48° 43′ 39.95″N, 3° 59′ 19.86″W) and Delfzijl, the Netherlands (53° 20' 50.34″N, 6° 54′ 20.98″E).

RNA was extracted and cDNA synthesized as described in Pearson *et al.* (2006). Sequences (ABI 3130 XL, Applied Biosystems, Inc., Foster City, CA, USA) were assembled using CodonCode Aligner (v. 1.6.3; CodonCode Corporation, Dedham, MA, USA). SNPs were identified by PolyPhred v. 6.18 (Montgomery *et al.*, 2008) and confirmed by manual inspection of alignments. Primer design and genotyping were performed at the Spanish National Genotyping Centre's facilities at the University of Santiago de Compostela. Assay Design 3.1 (SEQUENOM®, San Diego, CA, USA) was used to generate multiplexed SNP assays (iPLEX). The 35 assays were contained in three multiplexes (Table 1). As recommended by SEQUENOM®, the eXTEND software was used to increase the likelihood of assay success and reduce possible genotyping errors. This includes mapping proximal SNPs (ProxSNP) in the Assay Design input files to ensure primer and amplification specificity (Gabriel *et al.*, 2009), and post-design screening of multiplexed primers for cross-binding (PleXTEND).

For testing marker variability across different spatial scales, samples were collected at 16 localities (Table 2 and Figure 1), representing both large geographical separation (e.g. between Portugal or Morocco and populations towards the centre of the species' range, in Cornwall, south-west England) and local-scale separation within particular regions. Genomic DNA was extracted using the NucleoSpin® 96 Plan kit (Macherey–Nagel, Düren, Germany) from silica-conserved apical vegetative tissue. Final DNA elutions were quantified spectrophotometrically (NanoDropTM 1000; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to provide at least 10–20 ng μL^{-1}. Genotyping was performed by the MassARRAY® SNP genotyping system (SEQUENOM®), following the manufacturer's instructions. The 200-short-cycle PCR amplification programme uses two cycling loops, one of five cycles that sits inside a loop of 40 cycles. Samples were denatured at 94°C, strands were annealed at 52°C for 5 s and extended at 80°C for 5 s. The annealing and extension cycle was repeated four times for a total of five cycles and then looped back to a 94°C denaturing step for 5 s before entering the five-cycle annealing and extension loop again. The five annealing and extension steps with the single denaturing step were repeated to reach a total of 40 cycles, with a final extension step at 72°C for three minutes. Polymorphisms were determined by mass spectrometry analysis with the Bruker Autoflex MALDI-TOF (Bruker Daltonics, Billerica, Massachusetts, USA). Spectral output was analysed using MassARRAY® Typer 4 software (SEQUENOM®).

Allele frequencies, observed (H_O) and expected heterozygosity (H_E), and F_{ST} (Wright, 1969), as well as linkage disequilibrium, were calculated in Arlequin version 3.5.1.2 for Linux (Excoffier & Lischer, 2010). Probabilities of departure from Hardy–Weinberg equilibrium (HWE) were calculated using the exact test method performed in Genepop version 4.0 (Rousset, 2008). Significance was tested using permutations (N = 1000). Probability tests for population differentiation, based on the distribution of alleles across samples were performed using the algorithm of Raymond & Rousset (1995), implemented in Genepop. Frequencies of null alleles were estimated based on the method of Brookfield (1996), using FreeNA software version 1 (Chapuis & Estoup, 2007). A neighbour-joining tree was calculated using Cavalli-Sforza & Edwards (1967) distances to illustrate the separation of populations at the different spatial scales used, using Populations software version 1.2.30 over 10 000 bootstraps on loci (http://bioinformatics.org/tryphon/populations/).

Results

A total of 728 individuals belonging to 16 populations were genotyped, resulting in a PCR amplification success that varied between 78–99% across loci, although three SNPs did not amplify for any individual from Widemouth Bay (WM), Cornwall (Table 2 and Fig. 1). The proportion of

Table 1. SNP markers designed for the brown alga *Fucus vesiculosus*. Marker nomenclature is derived from the original cDNA library (*fs*, *F. serratus*; *fv*, *F. vesiculosus*), followed by contig and locus identifiers. The best database match field shows putative gene annotation after Blastx searches in the NCBI and Swiss-Prot databases. Flanking primers for initial gene amplification, primers for single base extension and the multiplex set (Iplex^{TM}; used to amplify several products in a single reaction) for each SNP locus are also shown.

Marker name	Best database match	Genome	Score	E-value	Iplex	Flanking primers (5′–3′)	Extension primers (5′–3′)
fs056_1	Photosystem II 12 kDa extrinsic protein	*Ectocarpus siliquosus*	204	2.0E-53	1	F: ACGTTGGATGTATCTGCAGCGGCGGTCTTT R: ACGTTGGATGAACATCGATCTTCTCGCCAC	F: CCCAGTGGTGCCATTC
fs116_1b	Conserved unknown protein	*Ectocarpus siliquosus*	263	6.2E-68	2	F: ACGTTGGATGTAAAAAAGCCGGCCGCTTTG R: ACGTTGGATGTTGATGAGGTTTGCGACGGG	F: CCGATTTGTACGCGACAACATC
fs117_1c	Cathepsin L-like proteinase	*Ectocarpus siliquosus*	349	1.0E-97	2	F: ACGTTGGATGATCTCCACAGTCTTTGGCAG R: ACGTTGGATGAGTCAGAGCCGGCGTATGAT	R: GCGTTGGCCAACCGAGGCGGGATCAAA
fs118_5	Cytochrome c oxidase subunit VIa	*Ectocarpus siliquosus*	139	3.0E-34	1	F: ACGTTGGATGATAGTCCGCGATCTTCCTTG R: ACGTTGGATGAAACCGTTCCCTTGGACTTG	R: GAGGTCCAGGAGAGAA
fs138_2a	Selenoprotein T	*Ectocarpus siliquosus*	207	2.0E-54	2	F: ACGTTGGATGATAACCGGGACGAAGAAGGG R: ACGTTGGATGTGGGTTCTTACGTCCAGATG	R: GGGGAGACGAGGGTACCGAAGATTAC
fs138_2b	Selenoprotein T	*Ectocarpus siliquosus*	207	2.0E-54	3	F: ACGTTGGATGCGTGTTTTTCTAGCGAGCTG R: ACGTTGGATGCTTTCGAGGTTTGGATCGAC	R: GAACGTGCCCTTAGTTTCTC
fs179_1c	Glutathione S-transferase	*Ectocarpus siliquosus*	154	4.0E-38	2	F: ACGTTGGATGCACGTTCATTGCTGGAACTG R: ACGTTGGATGTTGCCAAGAACAGTACCTCG	R: GAGGGCAGTGCTGGAACTGGATTATGAC
fv103_1a	Glutaredoxin	*Ectocarpus siliquosus*	139	7.0E-34	3	F: ACGTTGGATGACGTTCGGTACCGTGGATTG R: ACGTTGGATGCCAAGTATGAGCTTGTTGAG	R: CCGCCTTTCCGTCTTTCCG
fv103_3	Glutaredoxin	*Ectocarpus siliquosus*	139	7.0E-34	3	F: ACGTTGGATGGTGCGTGTTTTGCTCTGTAG R: ACGTTGGATGAACAAGCCAGCGAACAAGAC	F: ATCTGTAGAGTGCTGGTGGC
fv133_2	Light harvesting complex protein	*Ectocarpus siliquosus*	330	3.0E-91	1	F: ACGTTGGATGGATCTGTTGCACGAGGATTG R: ACGTTGGATGACGGGATCAACGAGAAGTGG	R: CCCTTGCCAGACGTCCGTGCTTA
fv135_1	Arf1, ARF family GTPase	*Ectocarpus siliquosus*	369	1.0E-103	1	F: ACGTTGGATGTGTGGAGGCACTACTACCAG R: ACGTTGGATGGACACGGTCTCTGTCATTAG	F: CCACCCTACTACCAGAACACACAA
fv151_1a	Elongation factor 1 beta	*Ectocarpus siliquosus*	162	3.0E-41	3	F: ACGTTGGATGGGTGATCTTGTCGAACAGAG R: ACGTTGGATGTCGACAGATCGCAGATCATC	R: CCCCCCGGTATCCCACGGTTTA
fv151_2	Elongation factor 1 beta	*Ectocarpus siliquosus*	162	3.0E-41	3	F: ACGTTGGATGGGTGATCTTGTCGAACAGAG R: ACGTTGGATGTCGACAGATCGCAGATCATC	R: CCCAACAGAGCCTTGAGGTCG
fv169_1b	Conserved unknown protein	*Ectocarpus siliquosus*	381	4.1E-104	2	F: ACGTTGGATGTTTCTGCCGCACTGCGTTTG R: ACGTTGGATGAAACCTACATCATCCCCCTC	R. CACTGCGTTTGGGTTTTTC
fv174_4	Peptidyl-prolyl cis-trans isomerase, cyclophilin-type	*Ectocarpus siliquosus*	201	5.0E-33	3	F: ACGTTGGATGCAGATAAAGAACTGCGAGCC R: ACGTTGGATGCTGATGTGAAGCACGACAAG	R: GAGCCATTTGTGTTCTTG
fv199_2	Conserved unknown protein	*Ectocarpus siliquosus*	151	3.7E-35	1	F: ACGTTGGATGAATCGGGATCGTGGGAGATG R: ACGTTGGATGTCCGCGAAGATGAGGATTAG	F: GGATCAGGAGAAGCTCTTTGTTGGA
fv209_2a	Conserved unknown protein	*Ectocarpus siliquosus*	302	5.0E-83	2	F: ACGTTGGATGCCTTCGACATTACCACTTCC R: ACGTTGGATGTGCTGGTTTTGACTTGAGCG	R: CCTTCCTGGAAGTCCCCCTTAGAGAAA
fv220_4	Conserved unknown protein	*Ectocarpus siliquosus*	257	4.8E-67	1	F: ACGTTGGATGTTTGGCCGGTATAGACTCTC R: ACGTTGGATGCGGTTATCACGGACGATTTC	R: GGGCGCACATGCCGTAGAGA
fv232_1c	Initiation factor 3H1 subunit	*Ectocarpus siliquosus*	407	1.0E-114	3	F: ACGTTGGATGGTTCCTCACGAATGTAGTGG R: ACGTTGGATGTTTTGAACACTCGCTCACCG	F: CCTAACGAATGTAGTGGTGGTTATA

fv232_2b	Initiation factor 3H1 subunit	*Ectocarpus siliquosus*	407	1.0E-114	3	F: ACGTTGGATGACTGCACCCAGATCAACAAG R: ACGTTGGATGTCGCTACGACTTCTGCAGAG	F: TTCGCGGGATCTTCCTTC	
fv232_4a1	Initiation factor 3H1 subunit	*Ectocarpus siliquosus*	407	1.0E-114	2	F: ACGTTGGATGCGGAGATTCTAGAAGAGCTG R: ACGTTGGATGCCTTCCTGAAGGTCAAAAAC	F: CCGATCAAGATCAGGAACCC	
fv251_1a	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase, chloroplast precursor	*Ectocarpus siliquosus*	546	1.0E-156	3	F: ACGTTGGATGACGTTCGAGGTTACTTGCAC R: ACGTTGGATGGGTGATAAGCAAGTTGAGGG	F: GGAGAGGTTTCGGTGATCATGCAAGTC	
fv251_1b	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase, chloroplast precursor	*Ectocarpus siliquosus*	546	1.0E-156	2	F: ACGTTGGATGAGAGTGTTTCAACACCTCGC R: ACGTTGGATGATTTCAGCAGCTCCTGGAAG	F: AAGCAACTACTGAGAGCGATCTG	
fv264_2	Mago nashi	*Ectocarpus siliquosus*	235	7.0E-63	3	F: ACGTTGGATGACCAAGCACTTTAGGTCCTG R: ACGTTGGATGTTCCAAGATCGGATCGCTAC	R: TCCTGGATTAGGTAGTAGAAG	
fv296_3b	Vacuolar proton pump D subunit	*Thalassiosira pseudonana*	398	8.3E-109	3	F: ACGTTGGATGAGATGTTGGAGCTCGCGTTC R: ACGTTGGATGTCCTGCTCTTTCAGCTTCAC	F: GGGAGGTGCACTTTGGATGCTTCTAC	
fv468_1a	Oxygen-evolving complex-related	*Arabidopsis thaliana*	59	1.9E-7	2	F: ACGTTGGATGGAATCCGGCAACTACAGATG R: ACGTTGGATGCAGCCAATCGCATAGATTTC	R: CAGATGCCGTTGCTAC	
fv468_1b	Oxygen-evolving complex-related	*Arabidopsis thaliana*	59	1.9E-7	3	F: ACGTTGGATGGGTGTCTCTGGAAGAGTAAG R: ACGTTGGATGCGAAGTCAGCAAGACCATTC	R: AGAGTAAGAGGCAATCATTC	
fv468_3	Oxygen-evolving complex-related	*Arabidopsis thaliana*	59	1.9E-7	1	F: ACGTTGGATGGTTATCGTGTACGTAAGCTC R: ACGTTGGATGAGAGCAGTAAAATCACCCCG	F: TACGTAAGCTCCGAGAAC	
fv532_1d	Glutathione S-transferase	*Ectocarpus siliquosus*	120	4.0E-28	2	F: ACGTTGGATGACTGGATGATGATGGAGAGG R: ACGTTGGATGGGCAGAGCCCAATATTTCAG	R: GCTGGAGAGGTTTTACCGAGTTAC	
fv532_5b	Glutathione S-transferase	*Ectocarpus siliquosus*	120	4.0E-28	3	F: ACGTTGGATGTTTACCGAGTTAGTCGGAGG R: ACGTTGGATGCCCTTACCGAGTCAATCTTG	R: GGGAGGAGGTCTCGGCTTTGA	
fv536_1	YihA2, YihA/EngB-like GTPase	*Ectocarpus siliquosus*	114	9.0E-27	1	F: ACGTTGGATGGAGTGCCAAATCAAGTAGTG R: ACGTTGGATGATAGCTCGACACACACTCTC	F: GGGGAGAAGGTTACGCCTCATGATC	
s008_1a	Ribosomal Protein L24	*Ectocarpus siliquosus*	255	2.0E-68	3	F: ACGTTGGATGAAATGGACAAGGACCGCAAG R: ACGTTGGATGATGTTGACATCGGTGTCCTC	F. GTGCGGCAAGCGCACTGGC	
s008_4	Ribosomal Protein L24	*Ectocarpus siliquosus*	255	2.0E-68	1	F: ACGTTGGATGCGAGCAATGTTATCTCTCGC R: ACGTTGGATGACACCGATGTCAACATGGCG	R: AGGACGCCGCAGGGAGATGA	
s008_6	Ribosomal Protein L24	*Ectocarpus siliquosus*	255	2.0E-68	1	F: ACGTTGGATGACATGTGCGGGAGTACGTTG R: ACGTTGGATGAGGACTATTTCGGTAAAGGG	R: GCCATCAAGAATCGAGCAACAAAAAC	
s011_1b	Translationally controlled tumor-like protein	*Arachis hypogaea*	109	7.8E-22	2	F: ACGTTGGATGATTCCTACTGTGATTGGGCG R: ACGTTGGATGTTTACGGCCTCCTCAGTATC	F: GGTTTGGCGGCGAGTGTCTACGAAC	

Table 2. Description of 35 SNP loci derived from *Fucus vesiculosus*. The SNP allele identities and frequencies for all 16 population samples are shown, together with the number of individuals per population (N) and the percentage of missing genotypes. Codon, description of the codon from the original gene sequence (na = not applicable is used to indicate non-coding regions); st, substitution type (synonymous/non-synonymous); aa, amino acid change; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient per locus and significant deviations from Hardy–Weinberg equilibrium (NS, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$); F_{ST}, global differentiation (Weir & Cockerham, 1984); Corr F_{ST}, corrected F_{ST} under hypothesis of null alleles; monomorphic loci, number of monomorphic loci per population.

						DG	DP	DT	HP	KB	LB	LR	LX	MM	MN	PG	PN	SW	TH	VC	WM					
Name	codon	st	aa	% missing	SNP	$N=44$	$N=82$	$N=41$	$N=42$	$N=45$	$N=37$	$N=27$	$N=25$	$N=44$	$N=38$	$N=37$	$N=38$	$N=64$	$N=45$	$N=55$	$N=64$	H_O	H_E	F_{IS}	F_{ST}	Corr F_{ST}
fs056_1	TT(C/T)	syn	–	0.05	C	0.961	0.994	0.963	1	0.989	0.941	1	1	1	1	0.875	0.973	0.951	0.989	0.991	0.992	0.040	0.042	0.046 NS	0.028	0.038
					T	0.039	0.006	0.038	0	0.011	0.059	0	0	0	0	0.125	0.027	0.049	0.011	0.009	0.008					
fs116_1b	AT(C/T)	syn	–	0.02	C	0.114	0.333	0.1	0.073	0.078	0.149	0.200	0	0.220	0.118	0.149	0.108	0.143	0.067	0.519	0.175	0.264	0.291	0.092 NS	0.106	0.103
					T	0.886	0.667	0.9	0.927	0.922	0.851	0.800	1	0.780	0.882	0.851	0.892	0.857	0.933	0.481	0.825					
fs117_1c	(A/T)TT	non syn	Phe–Ile	0.02	A	0.25	0.086	0.413	0.354	0.489	0.3	0.660	0	0.375	0.395	0.486	0.224	0.539	0.289	0.510	0.119	0.365	0.443	0.177 NS	0.131	0.127
					T	0.75	0.914	0.588	0.646	0.511	0.7	0.340	1	0.625	0.605	0.514	0.776	0.461	0.711	0.490	0.881					
fs118_5	TG(C/T)	syn	–	0.01	C	1	0.907	0.988	0.975	0.933	0.986	1	1	1	1	1	0.961	0.961	0.977	1	0.984	0.024	0.050	0.528***	0.022	0.058
					T	0	0.093	0.012	0.025	0.067	0.014	0	0	0	0	0	0.039	0.039	0.023	0	0.016					
fs138_2a	(C/G)TA	non syn	Leu–Val	0.02	C	0.716	0.835	0.775	0.756	0.711	0.771	0.889	0	0.707	0.622	0.851	0.662	0.742	0.867	0.300	0.831	0.290	0.409	0.291***	0.164	0.159
					G	0.284	0.165	0.225	0.244	0.289	0.229	0.111	1	0.293	0.378	0.149	0.338	0.258	0.133	0.700	0.169					
fs138_2b	C(G/T)A	non syn	Leu–Arg	0.02	G	0.239	0.525	0.488	0.214	0.289	0.306	0.043	0	0.058	0.365	0.459	0.257	0.211	0.512	0	0.336	0.327	0.410	0.202*	0.139	0.138
					T	0.761	0.475	0.513	0.786	0.711	0.694	0.957	1	0.942	0.635	0.541	0.743	0.789	0.488	1	0.664					
fs179_1c	na	–	–	0.06	A	0.659	0.697	0.910	0.819	0.911	0.797	0.857	1	0.925	0.934	0.944	0.838	0.927	0.511	0.942	0.625	0.232	0.301	0.229***	0.125	0.119
					G	0.341	0.303	0.090	0.181	0.089	0.203	0.143	0	0.075	0.066	0.056	0.162	0.073	0.489	0.058	0.375					
fv103_1a	(A/C)GG	syn	–	0.22	A	1	0.982	0.851	1	0.942	1	1	1	0.942	0.838	0.935	0.986	0.802	0.984	0.825	0.988	0.059	0.115	0.483***	0.075	0.113
					C	0	0.018	0.149	0	0.058	0	0	0	0.058	0.162	0.065	0.014	0.198	0.016	0.175	0.012					
fv103_3	na	–	–	0.09	C	0.598	0.600	0.645	0.663	0.636	0.714	0.296	1	0.598	0.569	0.519	0.658	0.476	0.600	0.410	0.625	0.434	0.484	0.101 NS	0.056	0.057
					T	0.402	0.400	0.355	0.338	0.364	0.286	0.704	0	0.402	0.431	0.481	0.342	0.524	0.400	0.590	0.375					
fv133_2	CT(T/A)	syn	–	0.03	A	0.213	0.309	0.15	0.4	0.333	0.236	0.417	0.980	0.205	0.278	0.25	0.405	0.143	0.125	0.750	0.221	0.351	0.435	0.192 NS	0.189	0.184
					T	0.788	0.691	0.85	0.6	0.667	0.764	0.583	0.020	0.795	0.722	0.75	0.595	0.857	0.875	0.250	0.779					
fv135_1	CA(G/A)	syn	–	0.01	A	0	0.079	0.085	0.012	0	0.068	0	0	0.056	0.066	0.027	0	0.023	0.033	0	0.016	0.064	0.062	−0.033***	0.023	0.022
					G	1	0.921	0.915	0.988	1	0.932	1	1	0.944	0.934	0.973	1	0.977	0.967	1	0.984					
fv151_1a	GT(T/A)	syn	–	0.05	A	0.023	0.056	0.162	0.095	0.107	0.100	1	1	0.186	0.207	0.279	0.039	0.208	0.238	1	0.041	0.172	0.370	0.535 NS	0.531	0.527
					T	0.977	0.944	0.838	0.905	0.893	0.900	0	0	0.814	0.793	0.721	0.961	0.792	0.762	0	0.959					
fv151_2	GT(C/A)	syn	–	0.08	A	0.693	0.508	0.321	0.679	0.422	0.597	0	0	0.415	0.162	0.148	0.618	0.323	0.329	0	0.476	0.364	0.472	0.229 NS	0.200	0.199
					C	0.307	0.492	0.679	0.321	0.578	0.403	1	1	0.585	0.838	0.852	0.382	0.677	0.671	1	0.524					
fv169_1b	(G/A)AA	non syn	Glu–Lys	0.06	A	0.341	0.174	0.025	0.175	0.211	0.106	0.476	0	0.098	0.026	0.086	0.088	0	0.091	0.622	0.297	0.145	0.164	0.117 NS	0.064	0.071
					G	0.659	0.826	0.975	0.825	0.789	0.894	0.524	1	0.902	0.974	0.914	0.912	1	0.909	0.378	0.703					
fv174_4	GG(C/A)	syn	–	0.08	A	0.128	0.107	0.026	0.179	0.08	0.100	0.093	0.760	0.022	0.057	0.328	0.118	0	0.100	0	0.164	0.178	0.218	0.184 NS	0.223	0.218
					C	0.872	0.893	0.974	0.821	0.92	0.900	0.907	0.240	0.978	0.943	0.672	0.882	1	0.900	1	0.836					
fv199_2	GG(G/A)	syn	–	0.04	A	0.113	0.184	0.012	0.138	0.011	0.03	0.458	0	0.044	0.039	0.014	0.194	0.016	0.105	0.189	0.075	0.310	0.338	0.081 NS	0.072	0.073
					G	0.888	0.816	0.988	0.863	0.989	0.97	0.542	1	0.956	0.961	0.986	0.806	0.984	0.895	0.811	0.925					
fv209_2a	GA(T/C)	syn	–	0.02	C	0.307	0.228	0.225	0.159	0.267	0.243	0	0.260	0.207	0.342	0.284	0.263	0.262	0.443	0	0.119	0.244	0.296	0.175 NS	0.166	0.161
					T	0.693	0.772	0.775	0.841	0.733	0.757	1	0.740	0.793	0.658	0.716	0.737	0.738	0.557	1	0.881					
fv220_4	AA(C/T)	syn	–	0.05	C	1	0.892	0.947	0.988	0.932	0.959	1	1	0.950	0.929	0.897	0.987	0.911	1	1	0.897	0.094	0.097	0.035 NS	0.028	0.035
					T	0	0.108	0.053	0.013	0.068	0.041	0	0	0.050	0.071	0.103	0.013	0.089	0	0	0.103					
fv232_1c	na	–	–	0.03	A	0.034	0.207	0.683	0.036	0.244	0.133	0.435	1	0.366	0.722	0.153	0.056	0.547	0.045	0.144	0.185	0.286	0.410	0.303 NS	0.318	0.312
					G	0.966	0.793	0.317	0.964	0.756	0.867	0.565	0	0.634	0.278	0.847	0.944	0.453	0.955	0.856	0.815					
fv232_2b	TT(C/T)	syn	–	0.01	C	0.614	0.598	0.939	0.679	0.789	0.622	0.435	1	0.818	0.921	0.716	0.716	0.968	0.593	0.160	0.556	0.358	0.433	0.171 NS	0.210	0.198
					T	0.386	0.402	0.061	0.321	0.211	0.378	0.565	0	0.182	0.079	0.284	0.284	0.032	0.407	0.840	0.444					

fv232_4a_1	CC(G/C)	syn	–	0.18	C	0.048	0.279	0.146	0.208	0.279	0.181	0	0.140	0.128	0.132	0.433	0.357	0.116	0.048	0	–	0.213	0.284	0.248***	0.088	0.089
					G	0.952	0.721	0.854	0.792	0.721	0.819	1	0.860	0.872	0.868	0.567	0.643	0.884	0.952	1	–					
fv251_1a	GT(G/C)	syn	–	0.02	C	0.116	0.165	0.098	0.071	0.044	0.043	0.022	0.95833	0.034	0.122	0.056	0.068	0.048	0.14	0.019	0.167	0.127	0.207	0.386***	0.270	0.247
					G	0.884	0.835	0.902	0.929	0.956	0.957	0.978	0.041667	0.966	0.878	0.944	0.932	0.952	0.86	0.981	0.833					
fv251_1b	CT(G/T)	syn	–	0.01	G	0.023	0	0.038	0.012	0.011	0.068	0	0	0.06	0	0	0.066	0.07	0.034	0	0	0.036	0.046	0.217***	0.022	0.045
					T	0.977	1	0.963	0.988	0.989	0.932	1	1	0.94	1	1	0.934	0.93	0.966	1	1					
fv264_2	AT(A/C)	syn	–	0.09	A	0.849	0.728	0.716	0.798	0.822	0.722	0.704	0.080	0.829	0.786	0.865	0.829	0.656	0.756	0.595	0.794	0.341	0.391	0.128 NS	0.109	0.108
					C	0.151	0.272	0.284	0.202	0.178	0.278	0.296	0.920	0.171	0.214	0.135	0.171	0.344	0.244	0.405	0.206					
fv296_3b	TA(C/T)	syn	–	0.01	C	0.909	0.900	0.988	0.927	0.889	0.917	1	1	0.977	1	0.946	0.868	0.960	0.884	1	0.833	0.113	0.129	0.121 NS	0.034	0.042
					T	0.091	0.100	0.012	0.073	0.111	0.083	0	0	0.023	0	0.054	0.132	0.040	0.116	0	0.167					
fv468_1a	(G/A)TA	non syn	Val–Ile	0.02	A	0	0.037	0.025	0	0.033	0.069	0	0	0.037	0.039	0.149	0.013	0.008	0.022	0	0.025	0.051	0.055	0.074 NS	0.033	0.037
					G	1	0.963	0.975	1	0.967	0.931	1	1	0.963	0.961	0.851	0.987	0.992	0.978	1	0.975					
fv468_1b	(A/C)GG	non syn	Arg–Lys	0.02	A	0.693	0.778	0.671	0.878	0.811	0.714	0.523	1	0.791	0.703	0.784	0.919	0.714	0.875	0.390	0.831	0.284	0.372	0.235***	0.093	0.085
					G	0.307	0.222	0.329	0.122	0.189	0.286	0.477	0	0.209	0.297	0.216	0.081	0.286	0.125	0.610	0.169					
fv468_3	AA(T/C)	syn	–	0.12	C	0.088	0.183	0.073	0.110	0.056	0.203	0.060	0	0.122	0.027	0	0.145	0.056	0.156	0.037	–	0.167	0.171	0.021 NS	0.037	0.033
					T	0.913	0.817	0.927	0.89	0.944	0.797	0.940	1	0.878	0.973	1	0.855	0.944	0.844	0.963	–					
fv532_1d	GA(C/G)	non syn	Glu–Asp	0.19	C	0.579	0.600	0.55	0.697	0.733	0.762	1	0.63636	0.323	0.528	0.707	0.719	0.295	0.56	1	0.547	0.385	0.477	0.193*	0.129	0.123
					G	0.421	0.400	0.45	0.303	0.267	0.238	0	0.36364	0.677	0.472	0.293	0.281	0.705	0.44	0	0.453					
fv532_5b	G(C/T)C	non syn	Ala–Val	0.05	C	1	0.919	0.947	0.964	1	0.971	0.659	0.750	0.966	1	0.939	0.972	0.982	1	0.608	0.95	0.078	0.142	0.448***	0.180	0.172
					T	0	0.081	0.053	0.036	0	0.029	0.341	0.250	0.034	0	0.061	0.028	0.018	0	0.392	0.05					
fv536_1	(T/C)TG	syn	–	0.03	C	0.313	0.300	0.024	0.118	0.022	0.286	0.630	1	0.011	0.081	0.171	0.105	0.031	0.558	0.792	0.311	0.239	0.399	0.400 NS	0.366	0.363
					T	0.688	0.700	0.976	0.882	0.978	0.714	0.370	0	0.989	0.919	0.829	0.895	0.969	0.442	0.208	0.689					
s008_1a	GG(C/A)	syn	–	0.01	A	0.045	0.031	0.098	0.073	0.022	0.095	0	0	0.023	0.039	0	0.081	0.016	0.267	0	0.071	0.091	0.102	0.108 NS	0.071	0.076
					C	0.955	0.969	0.902	0.927	0.978	0.905	1	1	0.977	0.961	1	0.919	0.984	0.733	1	0.929					
s008_4	na	–	–	0.07	C	0.431	0.325	0.386	0.329	0.267	0.319	0.391	0.65217	0.453	0.409	0.227	0.333	0.482	0.288	0.529	0.164	0.272	0.463	0.412***	0.044	0.052
					T	0.569	0.675	0.614	0.671	0.733	0.681	0.609	0.34783	0.547	0.591	0.773	0.667	0.518	0.713	0.471	0.836					
s008_6	na	–	–	0.19	G	0.303	0.162	0.316	0.237	0.364	0.310	0.023	0	0.346	0.292	0.234	0.208	0.339	0.326	0	–	0.327	0.362	0.098 NS	0.070	0.075
					T	0.697	0.838	0.684	0.763	0.636	0.690	0.977	1	0.654	0.708	0.766	0.792	0.661	0.674	1	–					
s011_1b	na	–	–	0.03	C	0	0.038	0.013	0.012	0	0.014	0	1	0.051	0	0	0.041	0.008	0.058	0	0.024	0.027	0.100	0.732***	0.673	0.599
					G	1	0.962	0.988	0.988	1	0.986	1	0	0.949	1	1	0.959	0.992	0.942	1	0.976					
			monomorphic loci			7	1	0	3	3	1	15	26	2	6	5	1	2	2	16	1					

Fig. 1. Locations of the 16 analysed samples: DG, Durgan; DP, Duckpool; DT, Dittisham; HP, Helford Passage; KB, Kingsbridge; LB, Lower Barn; LR, Lima River; LX, Lixus; MM, Merthen Manor; MN, Marina; PG, Paignton; PN, Porth Navas; SW, Sharpham Winery; TH, Thurlestone; VC, Viana do Castelo; WM, Widemouth.

monomorphic loci varied widely across samples (Table 2); fixed loci were more common in the southern populations (26 monomorphic loci in Lixus, 16 in Viana do Castelo and 15 in Lima River) compared with central populations (maximum of seven monomorphic loci in Durgan). Overall, H_O ranged from 0.024 to 0.434. Observed heterozygosities were >0.1 in 25 loci and at 10 of these they were >0.3 (Table 2). A source of apparent, but erroneous nucleotide variation can arise when using the SEQUENOM® MassARRAY technique. The SpectroTYPER software uses the assay design information to calculate the expected position of the correct analyte peaks in the spectra. However nucleotide variation outside the SNP site will cause the resulting spectra to contain unaligned peaks, reported as extra polymorphisms (Gabriel *et al.*, 2009). We also noted that three SNPs did not amplify in a single sample (Widemouth Bay), probably due to failure of the annealing step for the designed primers. This situation implies the accumulation of variable sites on regions adjacent to those particular markers, reporting extra-variability for that sample.

One SNP locus (*fv135_1*) showed significant heterozygote excess, while significant heterozygote deficiency was observed in 13 of 35 SNP loci. Significant heterozygote deficiency was observed in five of the eight non-synonymous SNP loci. This is often attributed to null alleles, in which a heterozygous individual is erroneously genotyped as a homozygote (Vignal *et al.*, 2002). However, comparison of observed allele frequencies and F_{ST} values with those corrected under the hypothesis of null allele presence suggest that this is unlikely to be a widespread cause of the observed heterozygote deficiencies (Table 2). Estimated null allele frequency was > 0.02 in only three loci (*fs138_2a*, *fv103_1a* and *s008_4*) and > 0.01 in 19 loci across less than three samples. Null alleles are unlikely to explain the 13 loci with significant heterozygote deficiency in our dataset and therefore must be due to natural population processes, such as non-random mating and/or population subdivision.

The combined SNP loci were able to significantly differentiate all samples except for two pairwise comparisons: Dittisham *vs.* Marina, and Helford Passage *vs.* Port Navas (data not shown). The neighbour-joining tree (Fig. 2) revealed large differences between central and southern localities, whereas samples from nearby locations (within Portugal and within England) were grouped together. Within the southern part of the *F. vesiculosus* range, Lixus (Morocco) was very distinct from the other southern samples (Portugal).

As expected for neighbouring SNPs identified within the same gene, significant linkage disequilibrium across populations ($p < 0.099$) was found between pairs of markers located at the following loci (see Table 1): *fs138_2a* and *2b* (selenoprotein), *fs232_1c* and *2b* (initiation factor 3H1 subunit), *s008_4* and *6* (large subunit ribosomal protein L24). Other loci located within the same gene also showed some significant disequilibrium values, but in less than half of the studied populations. No linkage disequilibrium was detected among the rest of the loci, except for some pairs of loci in a maximum of two populations, which can be expected to occur by chance.

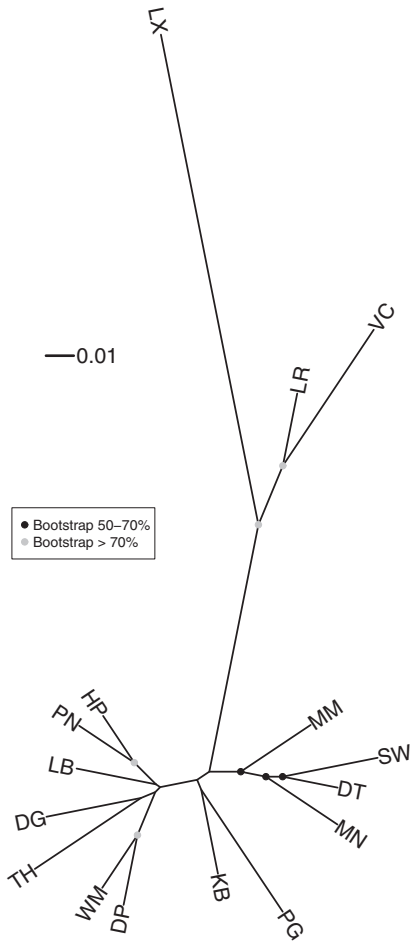

Fig. 2. Unrooted tree using neighbour-joining algorithm from Cavalli-Sforza & Edwards (1967) genetic distances. Node support is based on 1 000 000 bootstrap over loci. Genetic tree is scaled according to the scale bar.

Discussion

A set of 35 SNP markers has been developed that allowed us to successfully discriminate populations of *F. vesiculosus* at different spatial scales. An initial screening using the markers clearly shows southern range populations to be less polymorphic, with many more loci fixed for a particular allele than in central populations from the southwest of the UK (Table 2). This reveals lower diversity in the southern part of the range of *F. vesiculosus* compared with central populations, perhaps due to the small size and relative isolation of the former, although populations from Portugal and Morocco could still be easily distinguished by the markers (Fig. 2).

In recent years, SNP markers derived from ESTs have been used increasingly for estimating functional genetic diversity (Picoult-Newberg *et al.*, 1999). Rapid identification and verification of SNP markers using EST data sources is advantageous in leading to high-volume, cost-effective SNP discovery in plants (Kota *et al.*, 2003; Pavy *et al.*, 2006; Varshney *et al.*, 2007). One of the main advantages is that markers within or closely associated with coding regions can be identified (Picoult-Newberg *et al.*, 1999). Most of the *F. vesiculosus* cDNAs used here were annotated using the *Ectocarpus* genome (Table 1). We identified eight non-synonymous SNPs (i.e. resulting in amino acid substitutions), which are a potentially interesting marker class, since they may be linked with functional differences and phenotypic effects (Picoult-Newberg *et al.*, 1999).

Ascertainment bias, where markers are selected from an unrepresentative sample of individuals or genes, and therefore do not accurately reflect the allele frequency spectrum of the population(s), is potentially a significant problem when estimating and comparing linkage disequilibrium and population rates of migration, mutation and recombination (reviewed by Akey, 2003). First, the number of chromosomes sampled during the SNP design process, particularly if low, could affect linkage disequilibrium estimates. It is not currently possible to infer the chromosomal location of genes in *Fucus* due the absence of genomic information, e.g. a high-density genetic map. However, we carried out a linkage disequilibrium analysis based on a likelihood-ratio test with unknown gametic phase, where the likelihood of a particular sample is evaluated under the hypothesis of linkage equilibrium compared with an alternative hypothesis allowing association (Slatkin & Excoffier, 1996). For our SNP panel, the results suggest little effect of ascertainment bias on linkage disequilibrium estimates caused by low number of sampled chromosomes. The second cause of ascertainment bias is the population genetic characteristics of the particular genomic region considered (Akey, 2003). It is plausible that estimates of F_{ST} may vary across the genome (for example, selection could result in regionally restricted changes in effective population size), which may lead to a non-uniform distribution of ascertainment bias throughout the genome (Akey, 2003). Finally, ascertainment bias can arise as a consequence of gene flow and demographic history of the populations used for designing the markers (Akey, 2003). We used a broad resequencing panel of individuals from four populations, covering a large geographic scale (Iberian Peninsula to the Netherlands), and from rocky intertidal, soft substrate and estuarine (fluctuating salinity) environments, in order to prevent population and/or demographic effects which could affect the design.

Recent simulation studies carried out by Morin *et al.* (2009), indicated that ~30 SNPs should be sufficient to detect moderate ($FST \geq 0.01$) levels of differentiation, such as those previously reported for *F. vesiculosus* using similar markers

(Zardi *et al*., 2011), but also that sample size has a strong effect on the statistical power of the SNPs used. Morin *et al*.'s simulation results suggest that a data set of 50 specimens per sample should be more than adequate for defining evolutionarily significant units (ESUs) and will produce similar population parameter estimates as other highly variable markers, such as microsatellites. The proposed SNP panel fits these requirements. Differences in allele frequencies also have little effect on statistical power and markers can be relatively unbiased (Morin *et al*., 2009), although low frequency SNPs can provide complementary information for description at the population level (Brumfield *et al*., 2003). We describe 20 SNPs within 8 loci (2–3 SNPs per locus, see Table 1), which will increase the informative power of the panel by haplotype inference compared with the use of unlinked biallelic SNPs alone (Morin *et al*., 2009).

The SNP panel described here shows higher levels of expected heterozygosity than other SNP panels already designed for seagrasses (Ferber *et al*., 2008) and 27 of our SNP loci had expected heterozygosity levels similar to those reported in plants (Osman *et al*., 2003; Batley & Edwards, 2007; Yuskianti & Shiraishi, 2010). Some of the markers also showed levels of expected heterozygosity similar to those reported in microsatellite-based studies for the same species (Engel *et al*., 2005; Perrin *et al*., 2007; Tatarenkov *et al*., 2007; Billard *et al*., 2010).

These novel markers provide a foundation for new research on this widely distributed, ecological model species, and we anticipate that it will be possible to obtain valuable and detailed information to infer evolutionary processes at different spatial and temporal scales, a major focus of current studies (e.g. Billard *et al*., 2010; Coyer *et al*. 2011*a*). Furthermore, these markers will also be useful to study how micro-evolutionary selective forces and molecular adaptation act on genes along strong gradients of abiotic stress in the intertidal zone and across latitudinal gradients, providing clues about local adaptation and adaptive differentiation.

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