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Genomic scans detect signatures of selection along a salinity gradient in populations of the intertidal seaweed *Fucus serratus* on a 12 km scale

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ABSTRACT

Detecting natural selection in wild populations is a central challenge in evolutionary biology and genomic scans are an important means of detecting allele frequencies that deviate from neutral expectations among marker loci. We used nine anonymous and 15 EST-linked microsatellites, 362 AFLP loci, and several neutrality tests, to identify outlier loci when comparing four populations of the seaweed *Fucus serratus* spaced along a 12 km intertidal shore with a steep salinity gradient. Under criteria of at least two significant tests in at least two population pairs, three EST-derived and three anonymous loci revealed putative signatures of selection. Anonymous locus FsB113 was a consistent outlier when comparing least saline to fully marine sites. Locus F37 was an outlier when comparing the least saline to more saline areas, and was annotated as a polyol transporter/putative mannitol transporter — an important sugar-alcohol associated with osmoregulation by brown algae. The remaining loci could not be annotated using six different data bases. Exclusion of microsatellite outlier loci did not change either the degree or direction of differentiation among populations. In one outlier test, the number of AFLP outlier loci increased as the salinity differences between population pairs increased (up to 14); only four outliers were detected with the second test and only one was consistent with both tests. Consistency may be improved with a much more rigorous approach to replication and/or may be dependent upon the class of marker used.

1. Introduction

Detection of genomic signatures of selection is a key goal of evolutionary biology, as it identifies regions of the genome that are shaped by selection to different environments. One strategy in the identification of loci influenced by selection is to use multilocus neutrality tests or genome scans (Vasemägi et al., 2005; Storz, 2005). The genome scan approach identifies marker loci that are linked to selectively-relevant target loci through 'genetic hitchhiking' (Smith and Haigh, 1974). Genome scans assume that whereas all loci are influenced by genetic drift or migration, only a subset of loci respond to selection (Luikart et al., 2003; Orr, 1998). Detecting signatures of selection, therefore, involves identification of loci with atypical patterns of genetic variability (outliers) compared to the rest of the genome.

Genetic signatures of selection have often been detected using anonymous markers (unknown target gene and/or chromosomal location), such as simple sequence repeats (SSRs, or microsatellites) and amplified fragment length polymorphisms (AFLPs). In addition to anonymous markers, SSRs identified from the untranslated regions (UTR) of expressed-sequence-tag (EST) libraries are of great potential interest as they can be closely linked to selectively relevant target loci (coding sequences) via genetic hitchhiking and consequently, more likely to detect signatures of selection (Li et al., 2004; Bouck and Vision, 2007). Alternatively, genomic scans can be performed using AFLP techniques, which do not require prior sequence knowledge (especially useful for non-model organisms) and can provide hundreds of random markers (dominant) covering the entire genome (Bonin et al., 2006).

Genes of functional importance have been identified via genomic scans for some species of crop plants (Casa et al., 2005; Vigouroux et al., 2002) and only recently for some 'wild' or non-model species such as salmon (Vasemägi and Primmer, 2005), seagrasses (Oetjen and Reusch, 2007), sticklebacks (Mäkinen et al., 2008a), oysters (Murray and Hare, 2006), and cod (Nielsen et al., 2006). Tests for detecting outlier loci in genome scans require neutral genetic models and many (often >100) loci (but see Nielsen et al., 2006; Akey et al., 2002). Several tests have been developed to detect outliers, all of which have potential advantages and disadvantages (reviewed in Guinand et al., 2004; Beaumont, 2005). An increasing number of studies have applied

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more than one outlier analysis to the same data set, an approach that often reveals inconsistent results (e.g., Bonin et al., 2006; Vasemägi and Primmer, 2005; Oetjen and Reusch, 2007; Mäkinen et al., 2008a,b; Kane and Rieseberg, 2007; Oetjen et al., 2010). Confidence in the conclusions reached regarding selective effects is strengthened by replication in outlier testing to provide a more robust detection (Bonin et al., 2006; Oetjen and Reusch, 2007; Wilding et al., 2001; Campbell and Bernatchez, 2004).

Seaweed species in the genus *Fucus* are found on rocky intertidal shorelines ranging from high intertidal pools (where salinity fluctuates widely due to evaporation and rainfall) to shallow subtidal depths of several meters (where salinity levels, be they brackish or marine, are more stable), as well as in tidal marshes and the atidal and low salinity Baltic Sea (Lüning, 1990). Some species, such as *F. vesiculosus*, inhabit the entire salinity range, whereas others, such as the primarily subtidal *F. serratus*, commonly inhabit narrow fjords where salinity can vary widely along a spatial (kms) and/or temporal (hrs to daily) scale. Consequently, selection on various osmoregulatory pathways may be an important aspect of the successful radiation of the genus throughout the northern hemisphere intertidal region. Furthermore, as some species are adapted to a wide range of salinities, genomic studies will provide a greater understanding of osmoregulation in marine algae.

The dioecious *F. serratus* is a key foundation species along the lower intertidal/upper subtidal shores of Europe, ranging from the White Sea and outer Baltic Sea to the Iberian Peninsula, with introductions to Iceland and Nova Scotia (Lüning, 1990; Coyer et al., 2006; Hay and MacKay, 1887). Dispersal is highly limited as the negatively buoyant eggs are fertilized almost immediately after release and dispersal by rafting reproductive individuals is unlikely (Coyer et al., 2006 and references therein). Average annual expansion rates of 0.3 to 0.6 km^{-yr} (Coyer et al., 2006; Brawley et al., 2009) are consistent with panmixia from 0.5 to 2 km (Coyer et al., 2003).

In the present study, we compared populations of *F. serratus* over a strong salinity gradient ranging from nearly freshwater (3 psu) to fully marine (33 psu) along a spatial scale of 12 km in a narrow Norwegian fjord. We used anonymous and EST-linked microsatellites analyzed with seven outlier tests, in conjunction with AFLPs, to examine patterns of selection (via outlier loci) associated with the salinity gradient.

2. Materials and methods

2.1. Sample locations

Four populations were sampled at a 12 km scale in June 2007, in an unnamed southern extension of Kvænangen in northern Norway (Troms) (Fig. 1). The populations were separated by 3.5 to 4.5 km and located along an 11.7 km transect, ranging from a salinity of 2.7 psu at the innermost site (K1) to 6.0 psu (K2), 22.0 psu (K3), and 33.0 psu at the outermost site (K4), which faced the open sea. The outermost site was separated from the others by a narrow constriction with a large volume and velocity tidal flow. The innermost site (K1) was a small lagoon (ca 1300 ha) at the mouth of a small river and was isolated from the other sites by a very narrow inlet. The middle sites (K2 and K3) also were in a confined body of water (ca 2700 ha), separated from K4 and K1 by the narrow constrictions.

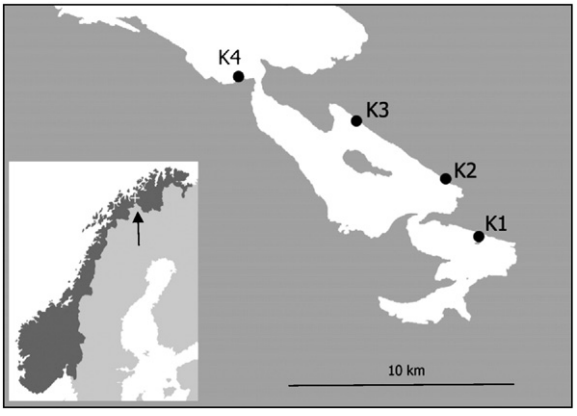

Fig. 1. Location of study sites K1–K4. Upper panel identifies location of study area (+).

2.2. Sample collection and DNA preparation

Apical tips (1–4) were excised from 50 individuals at ca. 1 m intervals along a transect line. Tips were blotted dry and placed in silica crystals for preservation and storage (Coyer et al., 2002). DNA was extracted and purified from 2 mg silica-dried tissue as described in Hoarau et al. (2007) and modified by Coyer et al. (2009).

2.3. Microsatellite genotyping

A total of 24 microsatellite loci were used in the analysis, nine anonymous (Coyer et al., 2002; Engel et al., 2003) and 15 from *F. serratus* and *F. vesiculosus* temperature and desiccation stress EST libraries (Table 1) (Coyer et al., 2009; Pearson et al., 2009). PCR reaction mixtures and conditions for the 9 anonymous loci (FsA198, FsB113, FsB128, FsD39, FsE9, FsF4, L20, L38, and L94) are described elsewhere (Coyer et al., 2002; Coyer et al., 2007) and for the 15 EST-derived loci (F9, F12, F17, F19, F34, F36, F37, F45, F47, F49, F50, F60, F65, F69, and F72) in Coyer et al. (2009). All genotypes were visualized on an ABI 3730 automatic sequencer (Applied Biosystems) and analyzed with GeneMapper v. 4.0 software (Applied Biosystems).

2.4. RACE

Three of the EST-derived loci (F9, F12, and F37) displayed outlier patterns according to our criteria (see discussion later), but could not be annotated. In an attempt in increase the length of sequence and the probability of meaningful annotation, we used RACE (rapid amplification of cDNA ends) on the three loci. Total RNA was extracted from powdered lyophilized *Fucus* tissue as described previously (Pearson et al., 2006), stored at −80 °C, and visualized on a 1.2% agarose gel to confirm integrity. RNA samples were digested with DNase I (RNase-free DNase set, QIAGEN) for 15 min at room temperature and purified using the RNeasy Mini kit (QIAGEN), according to the manufacturer's instructions. First-strand cDNA was synthesized for 5′-RACE from 1 μg of the purified total RNA using SMART RACE (Rapid Amplification of the cDNA Ends) cDNA Amplification Kit (Clontech), for 90 min at 42 °C, using a modified oligo(dT) primer (5′-RACE CDS Primer), the SMART II A oligo (Clontech), and PowerScript™ Reverse Transcriptase, according to the manufacturer's instructions. Gene-specific primers (GSPs) with a Tm of 68–72 °C were designed from *Fucus* EST sequence assemblies using Primer3 v. 0.4.0 (Rozen and Skaletsky, 2000). PCR amplification was performed with Advantage 2 polymerase mix (Clontech) using these GSPs, Universal Primer A Mix (UPM, Clontech) and 1–3 μl of the diluted first strand cDNA as template. Amplification products were analyzed on agarose gels, and, when present, stronger and/or larger molecular weight bands were excised and purified using EZNA gel extraction kit (OMEGA BIO-TEK), according to the manufacturer's instructions. The resulting DNA fragments were ligated into a pGEM®-T Easy vector (Promega) and the ligated vectors transformed into *Escheric. coli* DH-5α competent cells. Plasmid DNA was extracted from cloned cells by manual alkaline lysis (minipreps), quantified and sequenced with SP6 sequencing primer by chain-termination method, using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems), on a 3130xl Genetic Analyzer (Applied Biosystems).

Table 1
Pairwise comparison of populations and loci to determine outliers. A. Significance levels of three tests are shown in each cell from top to bottom: Beaumont F_{ST}-outlier test; Schlötterer tests (see text), ARLEQUIN Hierarchical SMM, ARLEQUIN Hierarchical IAM, and BAYESCAN. Anonymously-derived loci are preceded by Fs or L, EST-derived loci by F; loci putatively under selection indicated in gray. *= 95% level of significance, **= 99%, $^{+}$= at least 95% support for directional selection, $^{++}$= at least 99% support for directional selection, $^{\#}$= at least 95% support for balancing selection. Locus FsE9 did not amplify for one population and loci L58, F14, F21, F22, F58, and F59 were monomorphic; none of these seven loci were considered in the analysis. B. Significance test based on ARLEQUIN Hierarchial global test (K1K2/K3K4).

A	Comparison	FsA198	**FsB113**	FsB128	**FsD39**	FsE6	FsF4	**L20**	L38	L94	**F9**	**F12**	F17	F19	F34	F36	**F37**	F45	F47	F49	F50	F60	F65	F69	F72
	K1/K2	-	**	-	-	-	-	*	-	-	-	-	-	-	-	-	**	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	**	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
		+	-	-	-	-	-	-	-	-	-	++	-	+	-	-	-	-	-	-	-	-	-	++	-
	K1/K3	-	**	-	-	*	-	-	-	-	**	*	-	-	-	-	**	-	-	-	-	-	*	-	-
		-	**	-	-	-	-	-	-	-	-	**	**	-	-	-	**	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
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	K1/K4	-	*	-	*	-	-	**	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-
		-	-	-	*	-	-	-	-	-	-	*	**	*	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	#	-	-	#	-
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		-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
	K2/K3	-	-	-	-	*	-	-	-	-	**	*	-	-	-	-	-	-	*	-	-	-	**	-	-
		-	-	-	-	-	-	-	-	-	**	**	-	-	-	-	-	-	-	-	-	-	-	**	-
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	K2/K4	-	-	-	*	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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	K3/K4	-	-	-	**	-	-	**	**	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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		-	-	-	-	-	-	-	-	-	-	-	++	++	-	-	-	-	-	-	-	-	-	-	-
B	K1K2/K3K4 (SMM)																						+		
	K1K2/K3K4 (IAM)										+				#		+						+		

2.5. AFLPs

DNA concentrations and quality were examined with NANODROP 3.0.1 (NanoDrop Technologies). Digestion of 250 ng DNA with restriction enzymes *Eco*RI and *Mse*I, ligation of adapters, and PCR preamplification, were previously described (John et al., 2004), except for ligation, which was at 16° for 16 h (USB). Selective amplifications were performed using five primer pairs (Table 2) according to John et al. (2004), with the *Eco*-selective primers 5′ end-labeled with the fluorescent dye 6-FAM. Each primer-set was used for all samples at the same time and with the same mastermix. A MyCycler thermocycler (BioRad) was used for all PCR reactions. Fragments were separated by electrophoresis on an ABI 3730 automated sequencer (Applied Biosystems) and analyzed with GENEMAPPER v. 4.0 software (Applied Biosystems). AFLP settings for GENEMAPPER were: default base pair range = 100–250; bin width = 1.3; peak height acceptance, <50 RFU = discard, 50 RFU ≤ h < 100 RFU = check, h ≥ 100 RFU = accept; and peak amplitude thresholds for blue (FAM) dye = 250.

Table 2
Primer pairs used for AFLP procedure. Selective bases indicated in bold.

Primer pair	Sequence primer *Eco*RI	Sequence primer *Mse*I
1	5′-GACTGCGTACCAAATC + **AAG-**3′	5′-GATGAGTCCTGAGTAA + **CA-**3'
2	5′-GACTGCGTACCAAATC + **AGG-**3′	5′-GATGAGTCCTGAGTAA + **CA-**3'
3	5′-GACTGCGTACCAAATC + **AG-**3′	5′-GATGAGTCCTGAGTAA + **CAT-**3'
4	5′-GACTGCGTACCAAATC + **AAG-**3′	5′-GATGAGTCCTGAGTAA + **CC-**3'
5	5′-GACTGCGTACCAAATC + **AGG-**3′	5′-GATGAGTCCTGAGTAA + **CC-**3'

The AFLP loci were scored as present or absent. As sampling variance is high for markers with a low proportion of recessive phenotypes (band absence) in a given population, loci with <3% of band absence for all individuals were not scored (Bonin et al., 2006). To test for repeatability of bands, two independent DNA extractions were conducted for each of eight randomly selected individuals. Subsequent AFLP preparations and genotyping were done independently, but on the same day and with the same thermocycler. Differences in the banding patterns/scoring between replicates of each sample varied from 3% to 5%.

2.6. Data analysis

We defined loci (microsatellite and AFLP) putatively under selection as those loci with significant deviations in at least two of the statistical tests and in at least two population pairs. The population pairs also had to be meaningful, for example a comparison between pairs that consistently differed in salinity (e.g., K1/K4, but not K2/K3).

2.6.1. Microsatellites loci

The θ estimator of F_{ST} (Weir and Cockerham, 1984) and pairwise θ were estimated for microsatellites using GENETIX 4.02 (Belkhir et al., 2001). The significance of the pairwise comparisons was tested using 5000 permutations and sequential Bonferroni corrections, if necessary (Rice, 1989). The differentiation index D_{est} (Jost, 2009) was calculated for pairwise comparison between populations using the R software package DEMEtics (Jüterbock et al., 2010) (http://cran.r-project.org/web/packages/DEMEtics/index.htm). Bonferroni corrected p values (not shown) are based on bootstraps with 1000 repeats. The 24

microsatellite loci also were analyzed as active elements in a factorial correspondence analysis (FCA) using GENETIX 4.02 (Belkhir et al., 2001). FCA is a multivariate analysis that projects all individuals in a space defined by the components and each individual can be represented using each allele as an independent variable (She et al., 1987; Benzécri, 1973).

Seven neutrality model approaches were used for microsatellite outlier detection in all pairwise comparisons among K1, K2, K3, and K4. The first four approaches compare locus-specific heterozygosity H to the level of the fixation index F_{ST} resulting from pairwise population comparisons. Outlier loci were identified by unusually low or high F_{ST}/H ratios. The models used to generate a null distribution of F_{ST}/H ratios differ between the four approaches.

The first approach incorporated the finite-island model of Beaumont and Nichols (1996) (neutral genetic drift with migration between populations and mutation-drift equilibrium) using the software FDIST (url: http://www.rubic.rdg.ac.uk/~mab/software.html). An F_{ST} null sampling distribution is generated from coalescent simulations to identify outlier loci (unusually low or high F_{ST} values relative to a neutral drift model).

The second and third approaches, described in Excoffier et al. (2009) and implemented in ARLEQUIN v. 3.5 (Excoffier and Lischer, 2010) are similar to the first one in that they also use coalescent simulations assuming a finite-island model to obtain a null distribution around the observed values of pairwise population comparisons. The second approach differs from the third in that it assumes a stepwise mutation model (SMM) (Goldstein et al., 1995) instead of an infinite alleles model (IAM) and ρ_{ST} (Slatkin, 1995) is calculated instead of F_{ST} (calculated according to Weir and Cockerham, 1984).

In addition to pairwise comparisons of populations, we grouped the less saline (K1/K2) and the more saline (K3/K4) sites and compared them in a global test under the assumption of a hierarchical island model. We performed 20,000 simulations (defining 10 groups for the global test, 100 demes per group and expected heterozygosity ranging from 0 to 1). To obtain reliable results, number of groups and demes per group were intentionally set to a higher value than were empirically present (see discussion in Excoffier et al., 2009). Calculations were with both IAM and SMM models.

In our sixth approach, we used a Bayesian method (described in (Foll and Gaggiotti, 2008)) that is implemented in the software BAYESCAN (http://www-leca.ujf-grenoble.fr/logiciels.htm). In the analysis, two alternative models are defined (including/excluding the effect of selection) and their respective posterior probabilities are estimated using a Monte Carlo Markov Chain with the default settings.

The seventh approach utilized the so-called 'Schlötterer tests' (Schlötterer, 2002; Schlötterer and Dierienger, 2005). If variability at a neutral microsatellite locus is $\theta = 4N_e\mu$, a locus linked to a beneficial mutation will have a smaller effective population size (N_e) and consequently, a reduction in variability below neutral expectations. Because of differences in mutation rates (μ) among loci, however, a direct comparison is difficult and must be assessed by the relative variance in variability ($\ln R\theta$), which in turn can be estimated by the relative variance in heterozygosity ($\ln RH$) within-loci between-populations. The $\ln RH$ estimator of $\ln R\theta$ is expected to follow a normal distribution for neutral loci, consequently, outlier loci are putatively under selection (see Nielsen et al., 2006 for discussion and equations).

2.6.2. AFLP loci

Pairwise F_{ST} among AFLP loci was estimated using the software HICKORY (Holsinger and Lewis, 2007) to generate θ–II values that are directly comparable to the θ estimator of F_{ST} (Weir and Cockerham, 1984). The software does not provide P values for the pairwise tests, thus to determine if there was a significant differentiation, the test was conducted with different models (full model, $F_{IS} = 0$, and $F_{ST} = 0$ model). The model best reflecting the data is based on the deviance information criterion (DIC). The model with the smaller DIC is preferred, but a difference of less than 5 or 6 units among models indicates that there is no strong evidence favoring one model over another.

Outlier loci in the AFLP data set were tracked using two approaches. We first used the software DFDIST, which incorporates the Beaumont and Nichols model modified for AFLPs (Beaumont and Nichols, 1996, http://www.rubic.rdg.ac.uk/~mab/). The software estimates F_{ST} (Weir and Cockerham, 1984) between subgroups in the sample and also implements a Bayesian method developed by Zhivotovsky (1999) to estimate heterozygosity from the proportion of recessive phenotypes in the sample. DFDIST was run for each pairwise comparison of the four populations. Parameter conditions for DFDIST were: critical frequency = 0.99; Zhivotovsky parameters = 0.25; trimmed mean $F_{ST} = 0.3$ (excluding 30% of highest and 30% of lowest F_{ST} values; ca. estimate of average "neutral" F_{ST} uninfluenced by outlier loci); 50,000 resamplings; critical $P = 0.05$; and level of differentiation (target average θ) = 0.048. These parameters allow DFDIST to reliably estimate the proportion of the genome subject to selection as estimated by the proportion of outliers (Caballero et al., 2008). There was no trend in the proportion of p-values > 0.5 with increasing heterozygosity (data not shown) and the proportion of

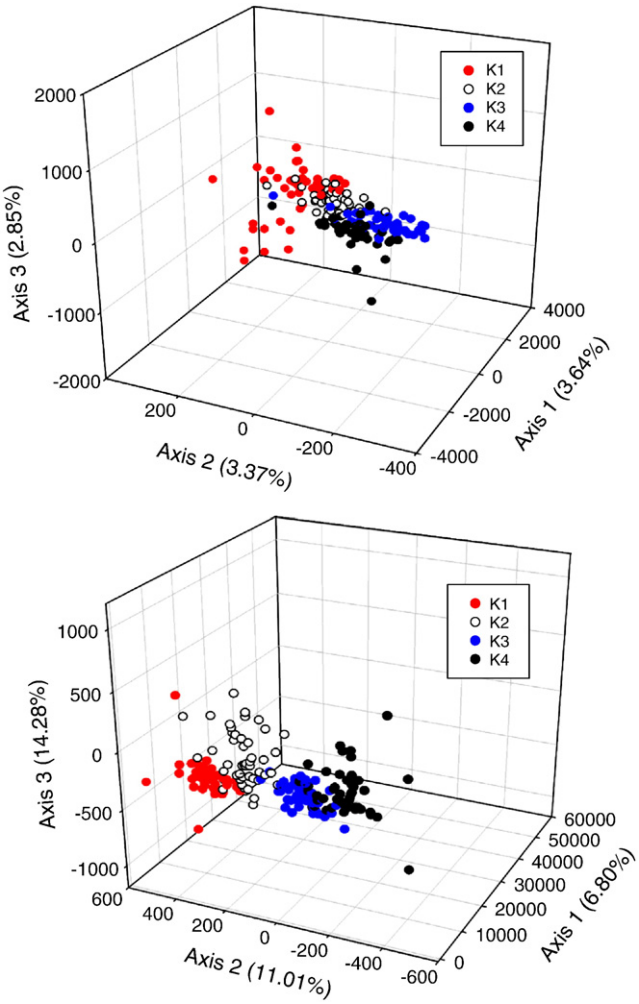

Fig. 2. Distribution of F_{ST} values as a function of heterozygosity for microsatellite (top) and AFLP (bottom) loci using Factorial Correspondence Analysis. Pairwise comparisons for the four locations differing in salinity are presented (K1 = 3 psu; K2 = 6 psu; K3 = 22 psu, K4 = 33 psu) (see Fig. 1).

Table 3
Pairwise F_{ST} and D_{est} for microsatellite loci. Upper, pairwise F_{ST} (θ estimator, Weir and Cockerham, 1984) for all 24 microsatellite loci (above diagonal, overall $F_{ST}=0.140$) and without the six outlier loci (FsB113, FsD39, L20, F9, F12, and F37) (below diagonal, overall $F_{ST}=0.097$). All pairwise F_{ST} were significant at $P=1.00$ (5000 permutations). Lower, pairwise D_{est} for all 24 loci (above diagonal, overall mean = 0.140) and without the six outlier loci (FsB113, FsD39, L20, F9, F12, and F37) (below diagonal, overall mean = 0.123). All comparisons were significant at $P=1.00$ (5000 bootstraps).

	K1	K2	K3	K4
K1	–	0.0880	0.1929	0.1996
K2	0.0553	–	0.0985	0.1221
K3	0.1355	0.0920	–	0.1152
K4	0.1131	0.1151	0.0997	–
K1	–	0.1033	0.2046	0.2231
K2	0.0778	–	0.0920	0.1151
K3	0.1799	0.0995	–	0.0997
K4	0.1416	0.1201	0.1035	–

p-values < 0.5 was equivalent to the proportion > 0.5 (data not shown); both characteristics imply a robust null hypothesis for neutral loci (see software notes, http://www.rubic.rdg.ac.uk/~mab/). Secondly, we used the software BAYESCAN (http://www-leca.ujf-grenoble.fr/logiciels.htm) with default settings (as above for microsatellites).

3. Results

Microsatellite analysis (FCA) distinguished each of the fjord populations (Fig. 2) as did the pairwise F_{ST} comparisons with or without the six outlier loci displaying signatures of selection (Table 3). Although the use of seven neutrality tests revealed discrepancies with regard to the number and identity of outlier loci, several patterns emerged according to our criteria (Table 1). Loci FsB113 and F37 were outliers when comparing the lowest salinity site (K1) to the more saline sites (K2, K3, and K4); F9 and F12 were outliers when comparing K1K2 with K3K4. Loci FsD39 and L20 were outliers when comparing the marine site (K4) with the lower salinity sites (K1, K2, and K3). A higher proportion of anonymous microsatellites (33%) revealed signatures of selection than EST-linked (20%).

Clinal variations in allelic frequencies were observed for five of the six outlier loci (Fig. 3). With the exception of Locus F12, allele frequencies of two alleles for each locus ranged from high to low or vice versa as salinity increased (K1 to K4). A similar pattern was detected for loci F60 and F65 that were not identified as outlier loci, but not for the other 16 loci (data available upon request).

None of the anonymous loci (clone sequences) could be annotated to six data bases (KEGG, KOG, SwissPro, *Thalassiosira pseudomona*

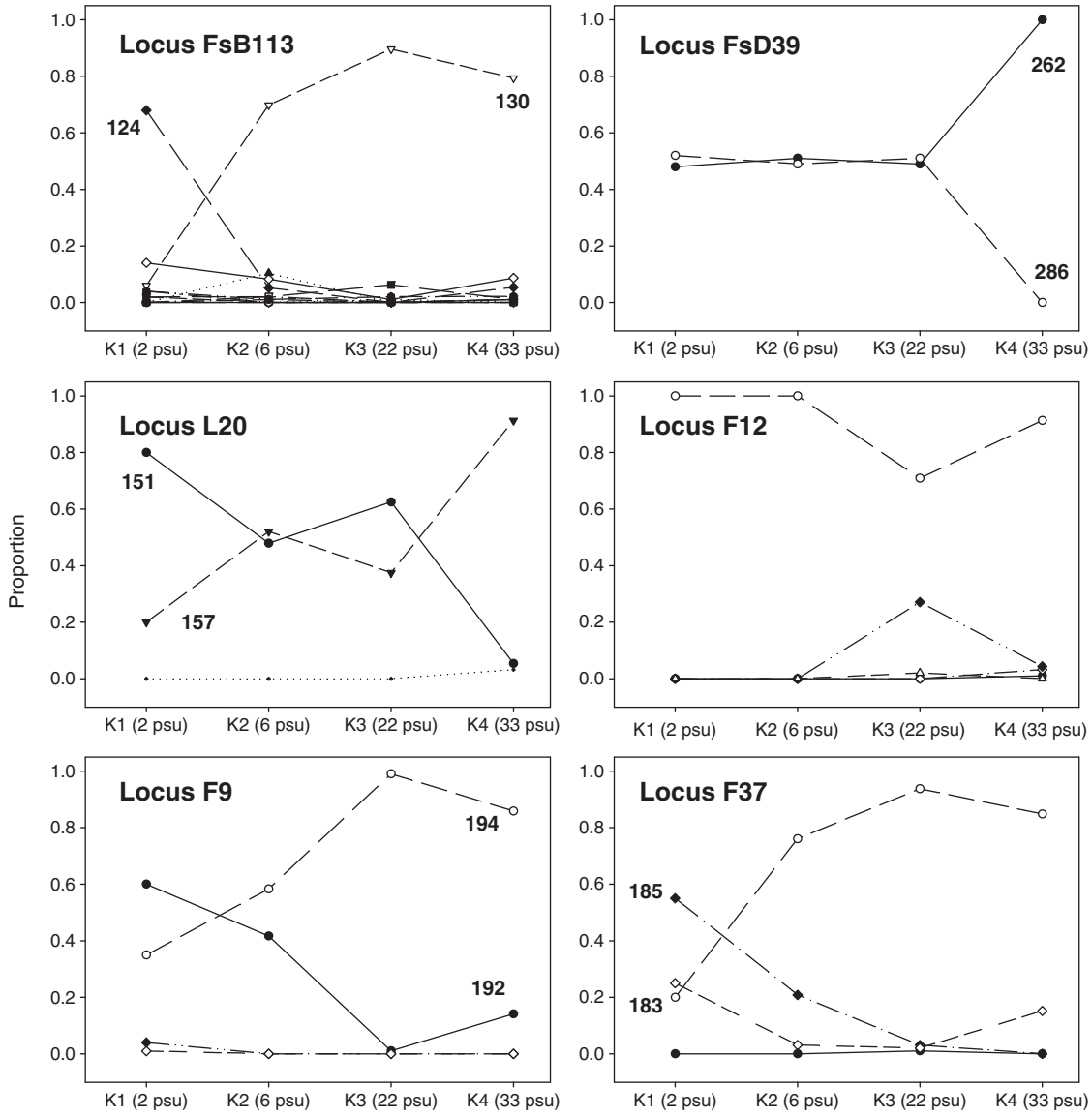

Fig. 3. Cline of allelic frequencies for six outlier loci across four populations.

Table 4
Pairwise F_{ST} (θ–*II* estimator, (Holsinger and Lewis, 2007)) for 362 AFLP loci. See text for discussion of P values. Overall F_{ST} = 0.1082. Only one outlier was common to both outlier tests, thus a comparison with and without the outlier was meaningless.

	K1	K2	K3	K4
K1	–	0.0516	0.1412	0.1067
K2		–	0.0893	0.0689
K3			–	0.0932
K4				–

genome, *Ectocarpus* ESTs, GenBank nt/nr). Application of RACE to the three EST-derived loci displaying outlier behavior (F9, F12, and F37), however, produced extended sequences and good annotation for one. Locus F37 had a strong homology (score = 103, E = $4e^{-20}$ against NCBI nr database) to an *Arabidopsis* polyol transporter/putative mannitol transporter.

A total of 188 individuals were scored for 362 AFLP markers, with each primer pair yielding 60 to 85 polymorphic AFLP bands. As for the microsatellite analysis, FCA of the AFLPs distinguished each of the fjord populations (Fig. 2). Pairwise comparison of F_{ST} values from AFLP loci using the full model in the software HICKORY suggested genetic differentiation and deviation from Hardy-Weinberg equilibrium for comparisons of K1–K2, K1–K3, K2–K3, and K3–K4 (Table 4). Where the $F_{IS} = 0$ model applies (comparison K1–K4 and K2–K4), there is evidence for genetic differentiation, but populations are in Hardy Weinberg Equilibrium. However, the $F_{ST} = 0$ model is not appropriate and does not apply for any of the pairwise comparisons as the DIC values are too large (compared to the other models) and consequently, provides evidence for all populations being genetically differentiated from one another.

The total number of outlier pairwise comparisons using AFLP markers in DFDIST was 46 of 362 (12.7%) and was positively correlated with the difference in salinity between pairwise comparisons (Figs. 4 and 5). For example, the number of outlier loci when comparing K1 (psu = 3) with K2 (psu = 6), K3 (psu = 22) and K4 (psu = 33) was 3, 13, and 14, respectively. Using BAYESCAN analyses and with the same criteria as for microsatellites, only three outliers were detected for the K1–K2 comparison (loci 140, 212, 236) and two in the K1–K3 comparison (locus 189 and 253) (data not shown). Only locus 253 was consistently identified as an outlier with both tests, although not in any other pairwise comparisons.

4. Discussion

During the process of population divergence and speciation, genetic differentiation accumulates in some regions of the genome (due to selection, genetic conflict, variable mutation rates, and chromosomal structure), but not in others (because of the homogenizing effects of gene flow and/or insufficient time for random differentiation by genetic drift) (Nosil et al., 2009, and references therein). The resulting and variable pattern of genomic differentiation

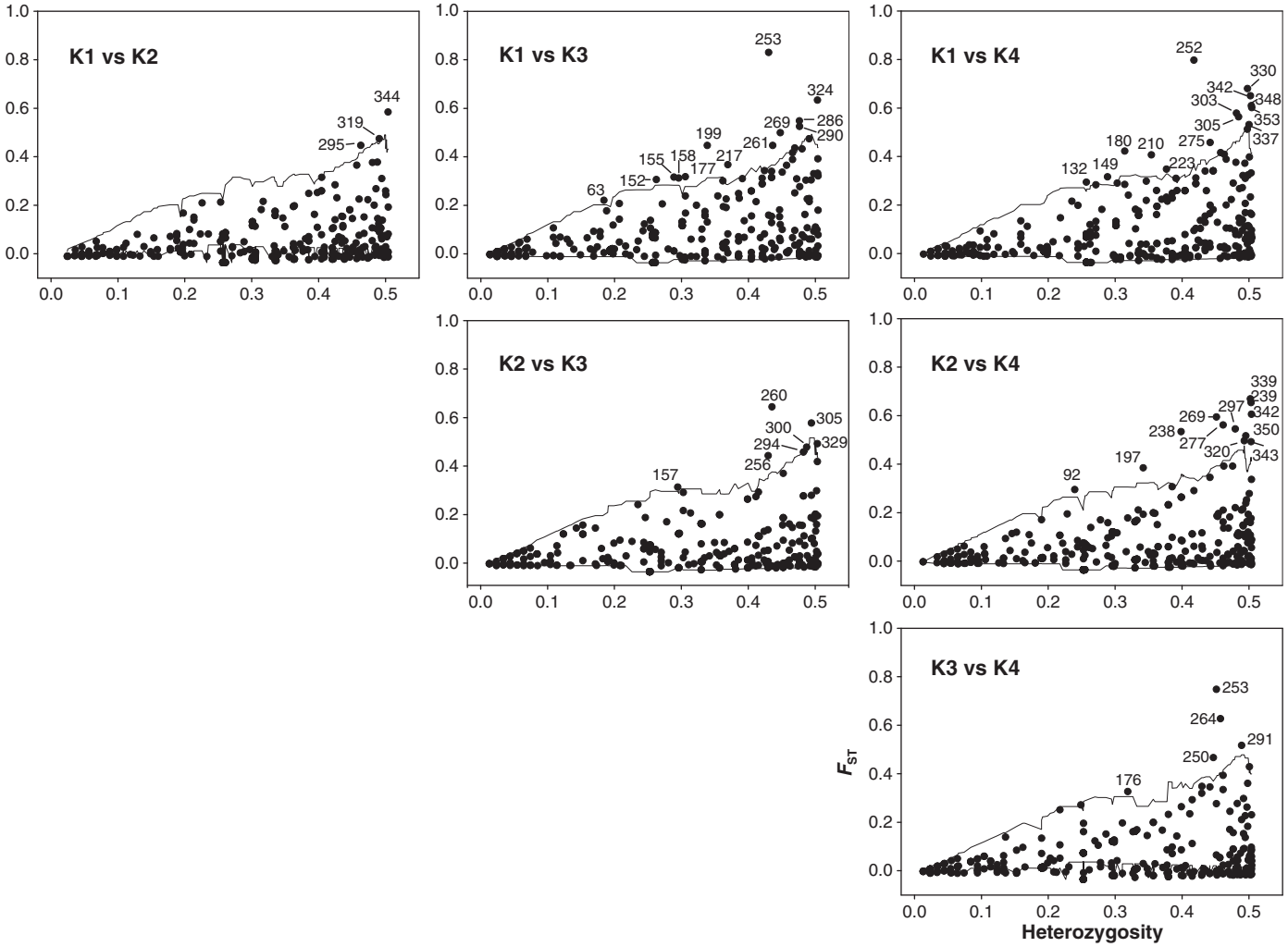

Fig. 4. Pairwise analysis of global F_{ST} vs allele frequency for 362 AFLP markers using DFDIST (see text). Outlier loci are identified; solid line represents 95% confidence limits. Salinities (psu) as in legend for Fig. 2.

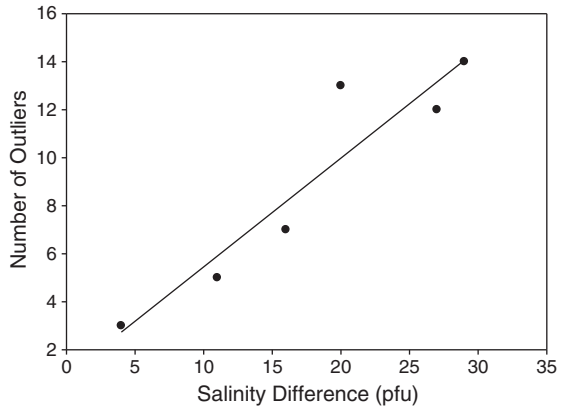

Fig. 5. Relationship between pairwise salinity differences and number of AFLP outliers among fjord populations. $R = 0.93$, $R^2 = 0.88$.

among populations has been referred to as 'heterogeneous genomic divergence' (Nosil et al., 2009, and references therein). Divergent selection, or selection acting in opposite directions in two populations, can contribute to heterogeneous genomic divergence by acting on specific loci (and those physically linked to selectively-relevant target loci via genetic hitchhiking) and by promoting reproductive isolation, which inhibits gene flow and facilitates genome-wide neutral divergence via genetic drift. The degree of differentiation observed among the four fjord populations of *F. serratus* (separated by ca. 3 to 12 km), particularly between the least (K1) and most (K4) saline populations, implies highly restricted gene flow and/or strong selection. Consequently, the fjord populations are excellent candidates for examining heterogeneous genomic divergence and divergent selection.

Gene flow may be restricted in part because of the broad range of salinity over which the populations exist and the importance of salinity in reproduction. For example, population K1 resided within a small lagoon separated from the other locations by a narrow constriction (Fig. 1). Consequently, the salinity encountered by K1 is likely to be low most of the time; whereas populations K2 and K3 may experience tidal fluctuations in salinity (K4 is fully marine). Although individuals in all four populations were morphologically similar, sexually mature individuals were observed only in populations K2, K3, and K4 (Coyer, unpub. data).

As sufficient salinity is essential for successful fertilization and germination in *Fucus* (e.g., Brawley, 1992; Serrão et al., 1999), the lack of sexually mature individuals in the K1 population (in June) severely restricts gene flow with populations K2, K3, and K4. Specifically, fertilization success in *F. serratus* decreases substantially with decreasing salinity: 87% at 9 psu to 5% at 6 psu (Malm et al., 2001). Additionally, growth rates of *Fucus* spp are more sensitive to low temperature if the salinity also is low (Munda and Kremer, 1997) and perhaps because of this relationship, fewer *F. serratus* eggs were released in a laboratory setting after the temperature permanently dropped below 4 °C (Malm et al., 2001). Thus, differences in salinity (and perhaps temperature) between K1 and the other locations could promote non-overlapping reproductive seasons between K1 and the others, leading to enhancement and maintenance of genetic differentiation in general, and the outlier patterns of microsatellite loci FsB113 and F37 in particular. Non-overlapping reproductive seasons among *F. serratus* populations are relatively common, as distinct summer and autumn reproducing populations (separated by ca. 60 km) have been reported in the brackish Baltic Sea (7–8 psu) (Malm et al., 2001). Thus, it is possible that the K1 population reproduces in autumn; an alternative hypothesis is that it reproduces only asexually, although asexual reproduction has not been observed in *F. serratus* (Malm et al., 2001). In either case, restricted gene flow with populations K2, K3, and K4 increases the relative importance of differentiation in K1.

The outlier EST Locus F37 was linked to an *Arabidopsis* polyol transporter/putative mannitol transporter and mannitol is one of the most commonly occurring sugar alcohol compounds. It is synthesized in bacteria, fungi, algae, lichens, and higher plants for a variety of functions including osmoregulation, as well as storage and scavenging of free radicals (reviewed in Iwamoto and Shiraiwa, 2005). Intracellular concentrations of mannitol increase in highly saline environments to control cell turgor in many organisms (reviewed in (Iwamoto and Shiraiwa, 2005)), including several species of brown algae (Munda, 1964; Reed et al., 1985). It has been shown that, while mannitol concentration and assimilation rate increased in the closely related species *F. spiralis* under hypersaline conditions, intracellular mannitol pools tend to be conserved under hyposaline conditions, in contrast to concentrations of major cations (Wright et al., 1989). This in turn, implies the presence of efficient transport mechanisms to sequester mannitol in intracellular pools during periods of low salinity, particularly when the salinity environment fluctuates. In light of this, putative selective pressure on genes involved in mannitol transport between population K1 and the aggregate of K2, K3, and K4 would be a reasonable expectation. Given the outlier designation of the mannitol transporter locus F37 and the putative reproductive isolation among population K1 and populations K2, K3, and K4 (= ecologically divergent), theory predicts strong selection on the locus (see Fig. 1 in Nosil et al., 2009). It is telling that two alleles of locus F37 reveal contrasting levels of relative abundance among the populations: allele 183 occurred much more frequently in populations K2–4, whereas allele 185 was much more abundant in population K1 (Fig. 3).

A similar outlier pattern was observed for locus FsB113. In contrast, the opposite pattern was observed for loci FsD39 and L20 (i.e., K4 differentiated from K1, K2, and K3). Unfortunately, none of these three anonymous loci could be annotated to any of the databases searched, as the length of sequence recovered from the clones of these anonymous loci was too short and attempts at extension using RACE were unsuccessful.

The proportion of outlier loci was lower for EST-derived loci (20%) than for anonymous-derived loci (33%). The deviation from the expectation that EST-linked loci would display relatively more outlier loci because of the higher probability of observing genetic hitchhiking with coding regions of the genome may be due to the small sample sizes of each category. Nevertheless, the values are comparable to proportions reported for gene-linked markers in seagrass, oak, and salmon (12–21%) (Vasemägi et al., 2005; Oetjen and Reusch, 2007; Scotti-Saintagne et al., 2004) and for anonymous-derived microsatellite loci in fish (18–23%) (Vasemägi et al., 2005; Mäkinen et al., 2008a; Nielsen et al., 2006). The proportion of AFLP outliers determined by DFDIST analysis (13%) also was comparable to values reported for frogs, leaf beetles, and holly leaf miner (8–18%) (Bonin et al., 2006; Scheffer and Hawthorne, 2007; Egan et al., 2008), but low for values determined by BAYESCAN (2%).

The use of putatively neutral genetic markers that have actually been targeted by selection to evaluate population structure may provide a biased view of relationships between populations. For example, populations of the intertidal snail *Littorina saxatilis* clustered by morphotype when 306 AFLP loci were used, but clustered by site when 15 loci identified as potentially under selection were removed from the analysis (Wilding et al., 2001). Similarly, a single microsatellite locus profoundly influenced genetic structure among cod populations (reviewed in Nielsen et al., 2006). For *F. serratus*, however, no differences in either the level or pattern of differentiation were apparent among populations when the six microsatellite loci were removed from the analysis (a similar analysis of the AFLP loci was confounded by the two outlier tests revealing only one outlier

locus in common). Consequently, the general pattern of differentiation in *F. serratus* may be relatively robust to inclusion of a few microsatellite loci putatively under selection and/or reflect fundamental differences that have arisen via genetic drift and affect all loci.

One of the main difficulties in identifying putative genes under selection in *F. serratus* is the low success rate of annotation, due in part to the great phylogenetic distance among lineages of brown algae (Heterokonts), as well as between heterokonts and plants/animals (Keeling et al., 2005), the latter of which comprise the majority of annotation data bases. However, the low level of annotation with two relatively closely-related heterokonts (the diatom *T. pseudomona* and brown multicellular algal *Ectocarpus siliculosus*) suggests that cDNA synthesis in *Fucus* is unable to recover a sufficient portion of the 5′ end of the transcript because the 3′ UTR is unusually long (Pearson et al., 2009). When cDNA transcripts were extended by RACE, however, significant and meaningful annotations for one of the three outlier loci were obtained. For *Fucus*, therefore, it is necessary to identify those EST loci displaying significant outlier patterns with two or more neutrality tests and extend the sequences with RACE in order to maximize annotation success.

Use of dominant AFLP markers offers another approach to detecting loci under selection and using the DFDIST analysis, the number of outlier AFLP loci was positively and significantly correlated with salinity differences (Fig. 4), Furthermore, some AFLP loci were outliers when comparing least with more saline populations and vice versa. Nevertheless, outlier determination of AFLP markers depends upon the choice of statistical test (as it did for the microsatellite loci), as the BAYESSCAN analysis revealed only five outlier AFLP loci with only one consistent between the two tests. Even if several statistical tests identify the same AFLP loci as outliers, obtaining gene annotations from AFLP loci may be even more difficult in the genus *Fucus* for a variety of technical reasons.

Our results suggest that whereas microsatellite-based approaches identified outlier loci that are putative genes under selection in the genus *Fucus* among populations along a steep environmental cline, a similar analysis of AFLP loci was less conclusive with larger discrepancies between the two different outlier tests. Preliminary data also indicates that the microsatellite approach can identify genes under selection among populations along a salinity gradient of >100 km spanning the Kattegat–Baltic Seas (data not shown).

5. Conclusions

Although the association of a putative mannitol transporter with a salinity gradient may be a logical expectation, it is prudent to realize that functional observations may not always have adaptive explanations (Gould and Lewontin, 1979; Nielsen, 2009). For example, outliers may be simply linked to the actual locus under selection (as opposed to being directly under selection) and the combination of a functional effect and selection does not demonstrate that selection has acted (Gould and Lewontin, 1979; Nielsen, 2009). Although the genomic scan provides an encouraging 'first result', direct evidence for selection on the putative mannitol transporter requires detailed experimental and population genetic approaches.

Several tests have been developed to detect outliers in genomic scan studies, and although many studies have applied more than one outlier analysis to the same data set, inconsistent results remain the norm, as they have in the present study. Some of the tests we used for the microsatellite loci identified the same loci as outliers, whereas others did not and similar results were obtained for AFLP loci. Consistency may be improved with a much more rigorous approach to replication and/or may be dependent upon the class of marker used.

Detailed characterization of the underlying mutations or genes under selection in the non-model organism *F. serratus* awaits solutions to the problem of adequate annotation and a fully sequenced genome. With the continuing development of 'next' generation high-throughput (HT) sequencing methods, the technical and financial feasibility of comparing experimentally-replicated, salinity-stress libraries under mesocosm conditions is likely to be more productive.

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