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Differential gene exchange between parapatric morphs of *Littorina saxatilis* detected using AFLP markers

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Abstract

Speciation requires the acquisition of reproductive isolation, and the circumstances under which this could evolve are of great interest. Are new species formed after the acquisition of generalized incompatibility arising between physically separated populations, or may they arise as a result of the action of disruptive selection beginning with the divergence of a rather restricted set of gene loci? Here we apply the technique of Amplified Fragment Length Polymorphism (AFLP) analysis to an intertidal snail whose populations display a cline in shell shape across vertical gradients on rocky shores. We compare the F_{ST} values for 306 AFLP loci with the distribution of F_{ST} estimated from a simulation model using values of mutation and migration derived from the data. We find that about 5% of these loci show greater differentiation than expected, providing evidence of the effects of selection across the cline, either direct or indirect through linkage. This is consistent with expectations from non-allopatric speciation models that propose an initial divergence of a small part of the genome driven by strong disruptive selection while divergence at other loci is prevented by gene flow. However, the pattern could also be the result of differential introgression after secondary contact.

Introduction

The process of speciation requires the acquisition of reproductive isolation. If populations are separated by a physical barrier to dispersal, speciation may follow: the acquisition of intrinsic reproductive isolation is then an incidental consequence of the accumulation of genetic differentiation (Mayr, 1963). Increasingly, attention has shifted to the possibility that reproductive barriers might arise in populations not separated by major physical features (Bush & Howard, 1986), i.e. that speciation might begin with genetic diversification in spite of some gene exchange between constituent populations. Empirical evidence shows, for example, that a single founder population in a lake may diversify and undergo speciation following use of different niches (Schliewen *et al.*, 1994; Schluter, 1996; Wilson *et al.*, 2000), and theoretical work suggests that gene flow can be less of a cohesive force than previously thought (Barton, 1988).

Barton (1988) and Rice & Hostert (1993) have reviewed the literature on speciation mechanisms, showing that there are plausible and simple models of non-allopatric speciation. In these models, genetic divergence may be initiated by disruptive selection without a period of extrinsic isolation. This requires strong selection and either pleiotropy or linkage of the genes involved in the adaptive polymorphism with those affecting the probability of gene exchange. For parapatric populations, where gene exchange is restricted, an initial level of differentiation may be modified to increase isolation by the accumulation of different alleles in the diverging genetic backgrounds. Strong selection also is needed here if gene flow is other than negligible. Nevertheless, Rice & Hostert (1993) concluded that laboratory experiments on the development of isolation strongly support the idea that

reproductive isolation can evolve between sympatric or parapatric populations if divergent selection is strong relative to gene flow.

Although the conclusion of Rice & Hostert (1993) is well supported by laboratory experiments, there is less evidence from natural populations. Host races provide the best examples, especially *Rhagoletis* (Feder *et al.*, 1994; Feder *et al.*, 1997). Host fidelity provides the major barrier to gene exchange, permitting further differentiation under selection on the alternative hosts. Some markers (presumably those linked to selected loci, or perhaps under selection themselves) show allele frequency differentiation, while others do not, suggesting that gene exchange is more restricted in some parts of the genome than others. This may be viewed as a signature of non-allopatric speciation and is in contrast to the generalised barrier to gene flow that results from physical isolation. The uniform divergence across the genome that evolves in allopatry may be maintained following secondary contact due to the accumulation of genetic incompatibility at many loci that is revealed in some hybrid zones (Barton & Hewitt, 1981; Szymura & Barton, 1991). However, it may be eroded by introgression.

We address the issue of uniform versus restricted differentiation using a system where divergent populations are parapatric. They are likely to be exchanging genes only in the region of contact, and the selection gradient on which they exist is imposed by the physical environment and by predation. *Littorina saxatilis* (Olivi) (the 'rough periwinkle') is widespread on North Atlantic shores, exhibits high morphological and allozyme variability, and is ovoviviparous and of low vagility - see Reid (1996) for review. In Britain it is found as two morphological forms ('H' and 'M') (Hull *et al.*, 1996) that show good evidence of partial reproductive isolation. This interpretation was based on reduced fertility in females inferred to be hybrids, and is

supported by the observation of assortative mating (Hull, 1998; Pickles & Grahame, 1999). The observed differentiation could be attributed to secondary contact between populations that had been undergoing allopatric divergence. Alternatively we may be seeing divergence *in situ* due to strong selection, despite gene flow (Endler, 1977; Rice & Hostert, 1993). In either case, the current pattern of differentiation is probably maintained by a balance between gene flow and selection, where the selection is due, at least in part, to environmental pressures rather than genetic incompatibility.

Predation by crabs is thought to exert strong selection on periwinkle shell form (Heller, 1976; Raffaelli, 1978; Janson, 1983; Johannesson, 1986), and among molluscs more widely - see Vermeij (1987) for review. Both thickness and form of the periwinkle shell may vary adaptively in response to differing predation pressures, and inducible phenotypic responses are considered to be involved for thickness changes in at least some species (Trussell & Smith, 2000). However, there is abundant evidence that in *L. saxatilis* some of the variation is genotypic (Newkirk & Doyle, 1975; Grahame & Mill, 1993; Johannesson & Johannesson, 1996), and this is especially likely for shell shape. Because crab predation increases down the shore in most sites, clines in shell shape are often found (Grahame *et al.*, 1997). In the upper shore, *L. saxatilis* H are thin-shelled, wide-apertured animals with relatively low spires. This shape may come about simply as a result of the constraints on shell shape when the aperture is large (Clarke *et al.*, 1999) thus affording greater foot area (Grahame & Mill, 1986) for adhesion and leading to greater gravitational stability (Heller, 1976). Therefore, this is probably the optimum shape for maintaining a grip on wave or wind-affected substrates in the absence of crab predation. In the lower shore *L. saxatilis* M are thicker shelled, with relatively smaller apertures; these features are

likely to be adaptive in reducing the risk of crab predation (Johannesson, 1986; Boulding *et al.*, 1999).

Primary and secondary origins of clines are notoriously difficult to distinguish (Barton & Hewitt, 1985). Wilding *et al*. (2000) considered it probable that the current distribution of mitochondrial haplotypes in *L. saxatilis* in the British Isles indicated expansion from different glacial refugia. However, the distribution of the H and M forms is quite different from that described for these haplotypes (Wilding *et al.*, 2000), and Wilding *et al.* (2001) concluded that the current haplotype distribution was unrelated to whether populations were H or M morph. We tentatively suggest that the *L. saxatilis* H-M cline has evolved *in situ*.

Here we examine putative loci (hereafter, simply 'loci') revealed by the Amplified Fragment Length Polymorphism technique (AFLP) (Vos *et al.*, 1995) in samples from four locations on the coast of Yorkshire, England. We compare observed F_{ST} distributions across loci between populations of *L. saxatilis* H and M with F_{ST} distributions in within-morph comparisons, and with expected distributions. These expected distributions were derived from simulations of F_{ST} values in the absence of selection, using an approach analogous to that of Beaumont & Nichols (1996). We ask whether the barrier to gene exchange between H and M populations is uniformly effective across loci.

Materials and methods

Sampling

Periwinkles were collected from rocky shores at Thornwick Bay, Flamborough (British Grid reference TA 233724), Filey Brigg (TA 132815), Old Peak (NZ 982024) and Robin Hood's Bay (NZ 955055). The coast trends overall northwesterly in this region, the straight line distances between the sites are: Flamborough - Filey Brigg, 15 km (we estimate that 60% of the intervening shore represents suitable habitat for *L. saxatilis*); Filey Brigg - Old Peak, 26 km (80% suitable habitat); Old Peak - Robin Hood's Bay, 4 km (90% suitable habitat). At each site snails were collected from each of two locations (one in an area occupied by the H morph and one in an area occupied by the M morph, except at Robin Hood's Bay), individual snails were taken from an area of about 2 m^2. H and M animals were characterized on the basis of sample location and shell form (by eye), and only brooding females were used to avoid contaminating the H samples with specimens of *Littorina arcana* Hannaford Ellis (which lay eggs on the shore). Sampling locations were 5 m apart at Flamborough, 15 m apart at Filey, 300 m apart at Old Peak, and 75 m apart at Robin Hood's Bay. In the first three instances, these distances were dictated by the presence of workable abundances of the animals, the aim being to sample from H and M populations which were as close to one another as possible. At Robin Hood's Bay the samples were of M animals only, 75 m was chosen as a distance likely to be considerably in excess of migration distance (Janson, 1983).

DNA isolation

Genomic DNA was purified from head-foot tissue of individual *Littorina saxatilis* using a modified version of Winnepenninckx *et al*. (1993). Tissue was macerated in 300µl 60°C CTAB buffer (2% CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl pH 8, 0.2% β-mercaptoethanol) to which 20mg proteinase K was added and incubated at 60°C for 3-16hr. Subsequently, two extractions with chloroform:isoamyl alcohol (24:1) were performed, and the DNA further purified with Promega's Wizard DNA Clean-Up System following the manufacturer's instructions. Concentration was assessed by spectrophotometry and adjusted to 100 ng.µl^{-1}.

AFLP analysis

AFLP analysis was performed using a modified version of Vos *et al.* (1995). Adapter and primer sequences are given in Table 1. For each sample genomic DNA (500ng) was digested with 5U *Eco*RI (NEB) and 3U *Mse*I (NEB) in 25µl total volume of 1x NEB buffer #2 supplemented with 100µg.ml^{-1} BSA, for 3 hours at 37°C. Following enzyme inactivation at 65°C, 25µl of a solution containing 5pmol *Eco*RI adapter, 50pmol *Mse*I adapter, 200U DNA ligase (NEB) and 5µl 10x ligase buffer (NEB), was added and samples incubated for 16 hours at 16°C. Preselective PCRs were then performed on 5µl diluted ligation (1:9 with 0.1xTE) in 50µl volumes containing 200µM each dNTP, 25pmol *Eco*+(C/A) primer, 25pmol *Mse*+(C/A) primer, 1.5mM $MgCl_2$ and 1U *Taq* in manufacturer's buffer. PCR conditions were 20x(94°C 30secs, 56°C 1min, 72°C 1min). Selective *Eco*+3 primers were labeled in 0.5µl volumes containing 1xT4 PNK buffer, 0.2µl T4 PNK (Promega) 5ng *Eco*+3 primer and 0.1µl γ^{33}P ATP. Selective PCRs were undertaken in 20µl volumes containing 30ng *Mse*+3

primer (see Table 1), 5ng labeled *Eco*+3 primer, 200μM each dNTP, 1.5mM $MgCl_2$, 1x buffer (Promega) and 0.4U *Taq*. Cycling conditions in the first cycle were 94°C 30secs, 65°C 30sec, 72°C 1min with the annealing temperature reduced by 0.7°C over next 12 cycles, then 23x(94°C 30secs, 56°C 30sec, 72°C 1min). On completion, 20μl STOP solution (95% formamide, 10mM EDTA pH8.0, 0.025% w/v bromophenol blue, 0.025% w/v xylene cyanol) was added. AFLP products were separated on 6% polyacrylamide gels (Sequagel, Flowgen), for 2-2½ hours at 55W then fixed, and dried to the glass plate. Kodak Biomax MR-1 film was exposed to the gel for 48 hours. An initial study of reproducibility showed absolute consistency of banding patterns between repeated reactions. Subsequent monitoring where ≈5% reactions were repeated has confirmed this.

Table 1 about here.

Data analysis

Gels were scored manually for band presence/absence. The frequency of the band presence allele was estimated from the band presence/absence matrix for each sample as $p = 1-((N-C)/N)^{0.5}$ where N = sample size and C = number of individuals with the band. This calculation assumes Hardy-Weinberg genotypic frequencies and dominance of band presence over absence.

We wish to use the allele frequency data for H and M samples to distinguish two possibilities: 1. That all loci reflect mutation/drift/dispersal balance, perhaps influenced by some general intrinsic barrier to gene exchange between H and M populations, or 2. That strong differentiation is maintained by selection at some proportion of loci, against a background of less-differentiated loci. We followed the approach developed by Bowcock *et al*. (1991) and Beaumont & Nichols (1996) by using simulations to predict the expected distribution of differentiation across loci for

179 a given average divergence. Differentiation is measured by F_{ST}, calculated for each
180 locus by the method of Nei (1977) with the correction suggested by Nei & Chesser
181 (1983). Simulation is necessary because the distribution of F_{ST} across loci is
182 influenced by historical sampling in the natural populations (i.e. by genetic drift) and
183 by experimental sampling. Here there is the added complication that AFLP loci are
184 dominant and, therefore, the experimental sampling error of F_{ST} is greater for high
185 mean allele frequencies (of the 'presence' allele) than for low frequencies. This is
186 because the allele frequencies have to be estimated from the proportion of 'absence'
187 homozygotes and the errors are greatest when this proportion is low.
188 We have used a simple simulation of two populations of size N diploid individuals,
189 with mutation rate μ and migration rate m, per generation. Allele frequencies for 500
190 simulated bi-allelic loci were initiated with a uniform random distribution, equal in
191 the two populations and then allowed to drift for $10N$ generations. Samples of 50
192 individuals were then taken from each simulated population and mean allele
193 frequencies and F_{ST} values were calculated in exactly the same way as for the
194 observed data (with the band presence allele dominant to the absence allele). The
195 simulation was checked by comparing the F_{ST} calculated in this way with both the F_{ST}
196 expected from theory and the F_{ST} calculated from the whole simulated population (i.e.
197 without sampling effects). The theoretical F_{ST} was calculated from
198 $F_{ST}=1/[1+16Nm+16N\mu]$ since only two populations are considered and the mutation
199 rate may be high relative to the migration rate (see below) (Crow & Aoki, 1984). The
200 simulated values calculated from the whole population agreed precisely with this
201 expectation but the simulated sample values showed a consistent upward bias of
202 0.0093 over the range of values of Nm relevant to this study. This bias is consistent

with previous simulation studies using Nei's method for calculation of F_{ST} (Slatkin & Barton, 1989).

For each comparison between observed samples, *Nm* in the simulation was set to a value expected to return the observed mean F_{ST} allowing for the estimation bias. The simulation was then repeated 50 times to generate a total of 25000 values of mean allele frequency and F_{ST} (minus those loci that were monomorphic in the simulated samples, approximately 5%). Simulated mean F_{ST} values differed from observed means by up to 6.77% but were always higher, making the test for loci with unexpectedly high levels of differentiation conservative. Observed F_{ST} values were compared with the 0.99 quantile of the simulated values determined for each of 20 categories of mean allele frequency, because the distribution of F_{ST} values is expected to vary with mean allele frequency (see below and Fig. 1).

Results

Levels of polymorphism

A total of 306 fragments (loci) were scored from five primer combinations for 50 individuals per sample (Table 2). Additional, variable fragments could not be scored unambiguously and were not considered further. Levels of polymorphism were particularly high with 94.8% of loci polymorphic (a locus was considered polymorphic if at least one individual showed a variant pattern). There was some variation in the number of scorable loci per primer combination with the *Eco*+CTC-*Mse*+CGA yielding 43 polymorphic bands and *Eco*+CAG-*Mse*+CGA yielding 80.

Table 2 about here

This high level of polymorphism suggests a value for $N\mu$ of the order of 10^{-1}, using Kimura's (1968) formula for bi-allelic loci. This formula assumes symmetrical mutation, which may not be true for AFLP bands, and ignores the possible existence

of many loci that are monomorphic for the 'absence' allele. This may mean that $N\mu$ has been overestimated. We have used $N\mu = 0.1$ ($N = 10^3$, $\mu = 10^{-4}$) in the simulations reported below but other runs have demonstrated that neither the mean nor the variance of F_{ST} is sensitive to these parameters (as also observed by Beaumont & Nichols 1996). We have also run simulations with the mutation rate from presence to absence ten times greater than the reciprocal rate. This increases the proportion of loci monomorphic for the absence allele but has no effect on the distribution of F_{ST}.

Detection of differentiated loci

Ten loci had F_{ST} values higher than the 0.99 quantile of the initial simulation results for all three individual H-M comparisons. Since these loci are implicated as being under selection or linked to areas of the genome that are under selection, *Nm* was recalculated after their removal, simulations were repeated, and the data compared with new 0.99 quantiles. This process was carried out four times. At this stage, no further locus showed observed values of F_{ST} lying above the 0.99 quantiles in all three H-M comparisons, and 15 loci were identified as lying above the 0.99 quantile (Fig. 1). If the three H-M comparisons were independent, one would expect to see <<1 locus falling outside the 0.99 quantile in all three cases (0.01^3 x 306). However, gene exchange between sites potentially means that allele frequencies do not vary independently. Therefore, we repeated the analysis making the alternative extreme assumption that the three H samples come from one population and the three M samples from another. In this case, all 15 of the loci previously identified fell outside the 0.99 quantile (now based on sample sizes of 150).

In all three H-M comparisons on the same shore, the same 15 loci lie above the 0.99 quantile, together with a much smaller number of other loci whose behaviour is

erratic. In comparisons within morphs, mostly also between shores, there are fewer loci above the 0.99 quantile, they are nearer to this limit, and rarely are any of the 15 loci identified above involved (see Fig. 1).

Fig. 1 about here

Table 3 shows that when F_{ST} is calculated using all loci, values are usually higher for H-M comparisons than they are for H-H or M-M comparisons. The few within morph comparisons which are as large as the smallest between morph ones are from samples at or near the extremes of the sample range, e.g. Old Peak H-Thornwick Bay H (0.0318). Yet overall, F_{ST} seems to be independent of distance, thus the F_{ST} for H-M at Thornwick Bay is 0.0378 (spatial distance 5 m) while the values for H at Thornwick Bay compared with the two M samples at Robin Hood's Bay (distance 45 km) are 0.0350 and 0.0340. The lack of relationship between all F_{ST} values and linear distance is further suggested by a randomization test (Manly, 1996; Manly, 1997) (1000 permutations) when the value of P for association was 0.3690. However if F_{ST} is estimated after removal of the 15 loci considered to be differentiated between H and M (Fig. 2), there is evidence of association with distance, P = 0.0020. In the figure, and for the randomization tests, distance was transformed by taking base 10 logarithms, F_{ST} by taking $F_{ST}/(1- F_{ST})$ as recommended by Rousset (1997).

Table 3 about here

Two-sample randomization tests (Manly, 1996; Manly, 1997) were carried out on the F_{ST} data in Table 3 either when the values were calculated with, or without, the 15 loci considered as likely to be differentiated. For values including these 15 loci, the probability that within morph and between morph F_{ST} values were the same was P = 0.001. When these 15 loci were excluded from the F_{ST} estimates, this probability became 0.1450, indicating no difference between the two groups of F_{ST} estimates.

Mean F_{ST} values after removal of these 15 loci imply that *Nm* between H and M

morphs within shores is in the range 5.5 at Old Peak, 6.3 at Thornwick Bay and 308 individuals per generation (respective *Nm* values were 1.9, 2.0 and 3.9 before removal). *Nm* between M morphs at Robin Hood's Bay is estimated as infinity (F_{ST} = 0).

Genetic variation among *L. saxatilis* populations

Nei's genetic distances between samples of *L. saxatilis* H and M were used to construct a neighbour-joining tree (Fig. 3a). The three samples of *L. saxatilis* H form one cluster separated from the five samples of *L. saxatilis* M by the greatest internal branch length and with high bootstrap support. When we omitted the data for the 15 loci identified as potentially under selection from the three comparisons of *L. saxatilis* H and M, the revised tree showed radically altered structure (Fig. 3b). Now, instead of populations clustering by morphotype (H and M), they cluster by site, with Filey H and M clustering together, Old Peak H and M together, etc.

Fig. 3 about here

Discussion

This study asks whether the *Littorina saxatilis* H-M cline represents a general barrier to gene exchange or reflects divergence at a limited number of loci under selection. By generating a large number of marker loci using AFLPs, and using the analytical approach of Beaumont & Nichols (1996), we have identified at least 15 loci (from a total of 306 studied; 5%) that seem either to be under selection or (more likely) linked to loci that are. However, none of the 306 loci is implicated as under selection when two populations of *L. saxatilis* M are compared from the same shore (Robin Hood's Bay). It is interesting that our H-M comparisons show differentiation at these loci regardless of whether they are spatially widely separate (300 m at Old Peak) or close together (5 m at Flamborough). Within morph comparisons do not show such differentiation, and now there is evidence of isolation-by-distance. F_{ST} values for between morph comparisons are evidently higher than for within morph comparisons when all loci are considered. The F_{ST} values after removal of these exceptional loci are more nearly similar, but still imply that there is a general barrier to gene exchange between H and M populations that is greater than would be expected from their spatial separation.

Our simulation assumes free recombination among loci. In reality, this is clearly not the case with 300 loci randomly distributed across the genome. In the extreme, some AFLP bands may be allelic or very tightly linked and so their levels of differentiation will not be independent. This will be detectable in hybridizing populations because it will generate strong disequilibrium between differentiated loci. We are currently analyzing such populations. However, in the present analysis, any effect of linkage would apply equally to all comparisons and so cannot explain the difference in distribution of F_{ST} between H-M and within-morph comparisons.

313 Thus, while there are no fixed differences between morphs in any of the
314 populations we have investigated, in appropriate comparisons (H versus M
315 populations), there is a small group of loci which show considerable differentiation
316 against a background of a majority where differentiation is weak. We suggest that this
317 is the most striking aspect of the data reported above: that there is a consistent group
318 of loci apparently differentiated. This point is further supported by comparing trees in
319 which the samples group by morphotype when the differentiated loci are included in
320 the analysis, but by shore when they are excluded. From this we infer that the majority
321 of the AFLP loci are in mutation/drift/dispersal equilibrium, although we cannot
322 exclude the possibility of a general reduction in gene exchange between H and M
323 populations relative to populations of the same morph. Against this background, we
324 suggest that differentiation is being maintained for the small number of differentiated
325 loci by selection on the loci themselves, or on closely linked loci. These findings are
326 consistent with earlier work demonstrating morphological, ecological and behavioural
327 differences between *L. saxatilis* H and M (Hull *et al.*, 1996; Hull, 1998; Pickles &
328 Grahame, 1999) but imply that the genetic differences underlying these characters
329 involve only a small proportion of the genome. This is what would be expected in a
330 case of non-allopatric speciation in progress. However, it could also be the result of
331 differential introgression following secondary contact resulting in homogenization of
332 allele frequencies at all loci except those under selection, or closely linked to loci
333 under selection.

334 The H and M forms of *L. saxatilis* represent one of several cases of divergence in
335 shell shape in this species. Similar variation is reported for shores in Sweden (Janson
336 & Sundberg, 1983), where it is considered to be phenotypic. It has been shown that
337 some allozyme loci are under selection, or linked to selected loci, in Swedish

populations (Johannesson *et al.*, 1995a; Johannesson & Tatarenkov, 1997), although this has not been explicitly associated with shell form. On the Galician coast of Spain very different shell forms occur in populations between which there is some restriction of gene flow and evidence of selection on shell form (Johannesson *et al.*, 1995b; Rolán-Alvarez *et al.*, 1997). We do not have direct evidence of selection operating on H and M forms on the Yorkshire coast, but it seems reasonable to infer that it does. The findings from Britain and Spain suggest that a pervasive influence in habitat use and subsequent diversification in *L. saxatilis* is the vertical shore gradient. In turn, this suggests an unusually simple physical background (a spatially very restricted cline, limited by the extent of the intertidal zones occupied by the animals) against which to study speciation processes.

Whether the differentiation of the small proportion of loci between H and M is primary (the result of divergent selection) or secondary (the result of renewed contact), the main point is that differentiation is maintained for a small portion of the genome, while gene exchange continues to prevent divergence at the majority of loci. Detailed investigation of these loci in particular may provide important insights into the nature of the barrier between these two forms of intertidal snail, and into the evolution of barriers to gene exchange in general.

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Table 1. Adapters and selective primer sequences used for AFLP analysis.

Primer/adapter	
Adapters	
*Eco*RI	5'-CTCGTAGACTGCGTACC-3'
	3'-CATCTGACGCATGGTTAA-5'
*Mse*I	5'-GACGATGAGTCCTGAG-3'
	3'-TACTCAGGACTCAT-5'
Primers	Sequence (5′-3′)
Eco+1	
Eco+A	GACTGCGTACCAATTCA
Eco+C	GACTGCGTACCAATTCC
Mse+1	
Mse+A	GATGAGTCCTGAGTAAA
Mse+C	GATGAGTCCTGAGTAAC
Eco+3	
Eco+ACG	GACTGCGTACCAATTCACG
Eco+CAG	GACTGCGTACCAATTCCAG
Eco+CTC	GACTGCGTACCAATTCCTC
Mse+3	
Mse+AGT	GATGAGTCCTGAGTAAAGT
Mse+ATC	GATGAGTCCTGAGTAAATC
Mse+CAA	GATGAGTCCTGAGTAACAA
Mse+CGA	GATGAGTCCTGAGTAACGA
Combinations	
A	*Eco*+CTC-*Mse*+CGA
B	*Eco*+CAG-*Mse*+CGA
D	*Eco*+CAG-*Mse*+CAA
E	*Eco*+ACG-*Mse*+ATC
F	*Eco*+ACG-*Mse*+AGT

Table 2. Levels of polymorphism of scored AFLP markers.

	Primer combination					
Restriction site	A	B	D	E	F	Total
No. of variable bands	43	80	54	54	59	290
No. of fixed bands	0	3	8	1	4	16
Total	43	83	62	55	63	306

Table 3. F_{ST} (below diagonal) between populations of *Littorina saxatilis* (mean over 290 loci). Above diagonal, F_{ST} following removal of 15 loci. Standard errors of F_{ST} estimates range from 9.80-22.15% (below diagonal) and 10.19-22.20% (above diagonal) of the mean. TH, Thornwick Bay; OP, Old Peak; FY, Filey Brigg; RB, Robin Hood's Bay (two samples, M only).

	TH-H	TH-M	OP-H	OP-M	FY-H	FY-M	RB1	RB2
TH-H		0.0190	0.0311	0.0372	0.0242	0.0227	0.0301	0.0298
TH-M	0.0378		0.0397	0.0293	0.0322	0.0217	0.0260	0.0255
OP-H	0.0318	0.0633		0.0204	0.0223	0.0256	0.0254	0.0247
OP-M	0.0489	0.0292	0.0396		0.0309	0.0241	0.0221	0.0212
FY-H	0.0247	0.0551	0.0238	0.0480		0.0095	0.0204	0.0207
FY-M	0.0328	0.0236	0.0402	0.0244	0.0247		0.0148	0.0142
RB1	0.0350	0.0308	0.0347	0.0241	0.0286	0.0156		0.0055
RB2	0.0340	0.0299	0.0339	0.0230	0.0291	0.0153	0.0052	

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Figure legends

Fig. 1

F_{ST} values estimated from 290 variable AFLP loci plotted against mean allele frequency in three H-M comparisons (Thornwick Bay, Old Peak and Filey), one M-M comparison (Robin Hood's Bay) and six representative between shores, within morph comparison. The solid line represents the 0.99 quantile estimated from a simulation model (see methods). The position of each of the 15 loci implicated as linked to a region under selection due to their presence outside the region defined by the 0.99 quantile consistently in all three H-M comparisons (see Results) is labeled with the locus identifier (where A-F = primer combination, see Table 1).

Fig. 2

Relationship of mean F_{ST} with linear distance, 15 differentiating loci removed.

Fig. 3.

Neighbour-Joining tree calculated from allele frequency data in PHYLIP (Felsenstein, 1993). Data were bootstrapped (x100) using SEQBOOT and Nei's genetic distance calculated using GENDIST. Distances were clustered with NEIGHBOR and the consensus tree constructed with CONSENSE. Bootstrap values are shown at the nodes of a representative non-consensus tree in order to retain branch length information. a), using all 290 AFLP loci; b), after removal of 15 differentiated loci.

Fig. 1

Fig. 2

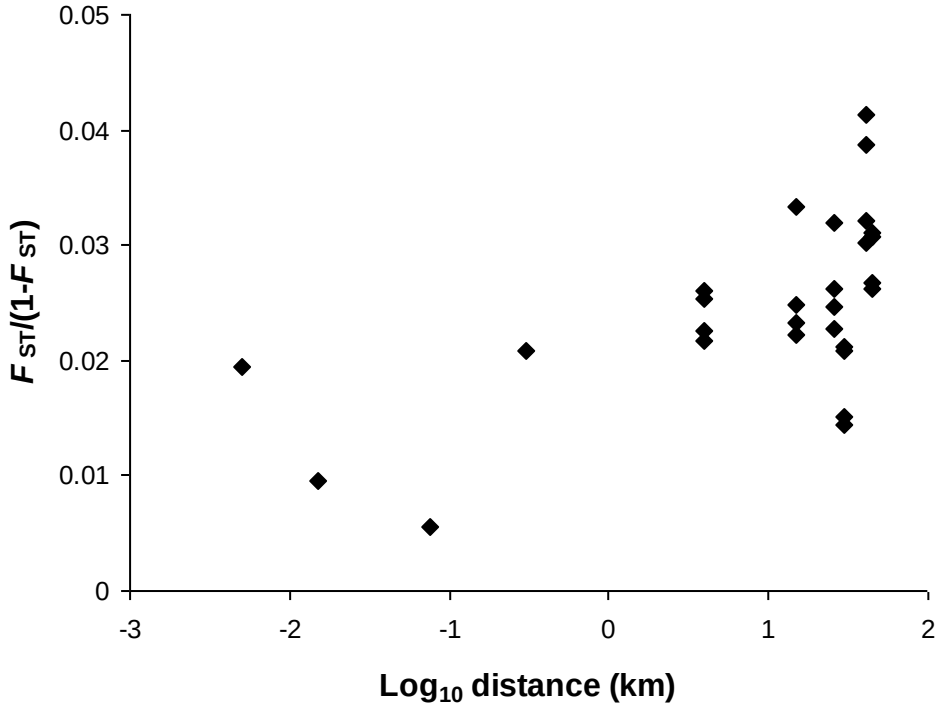


Fig. 3.

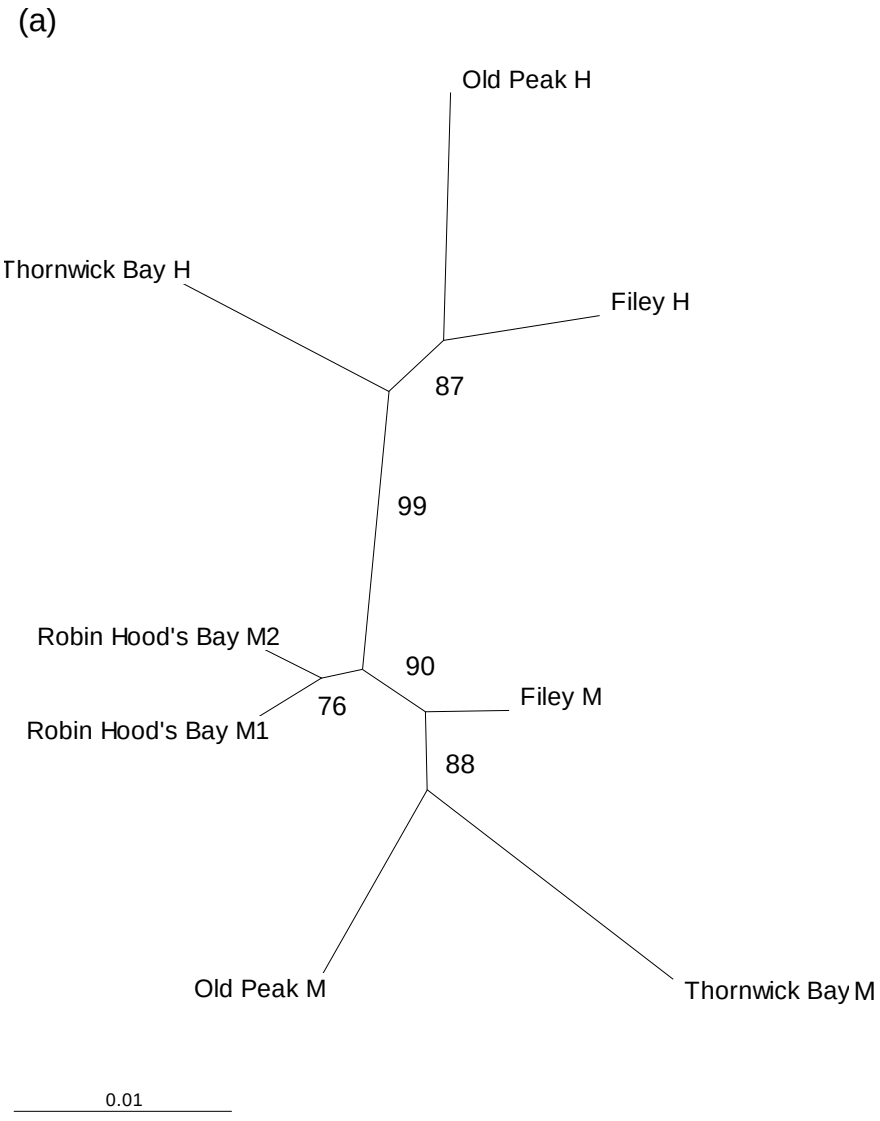
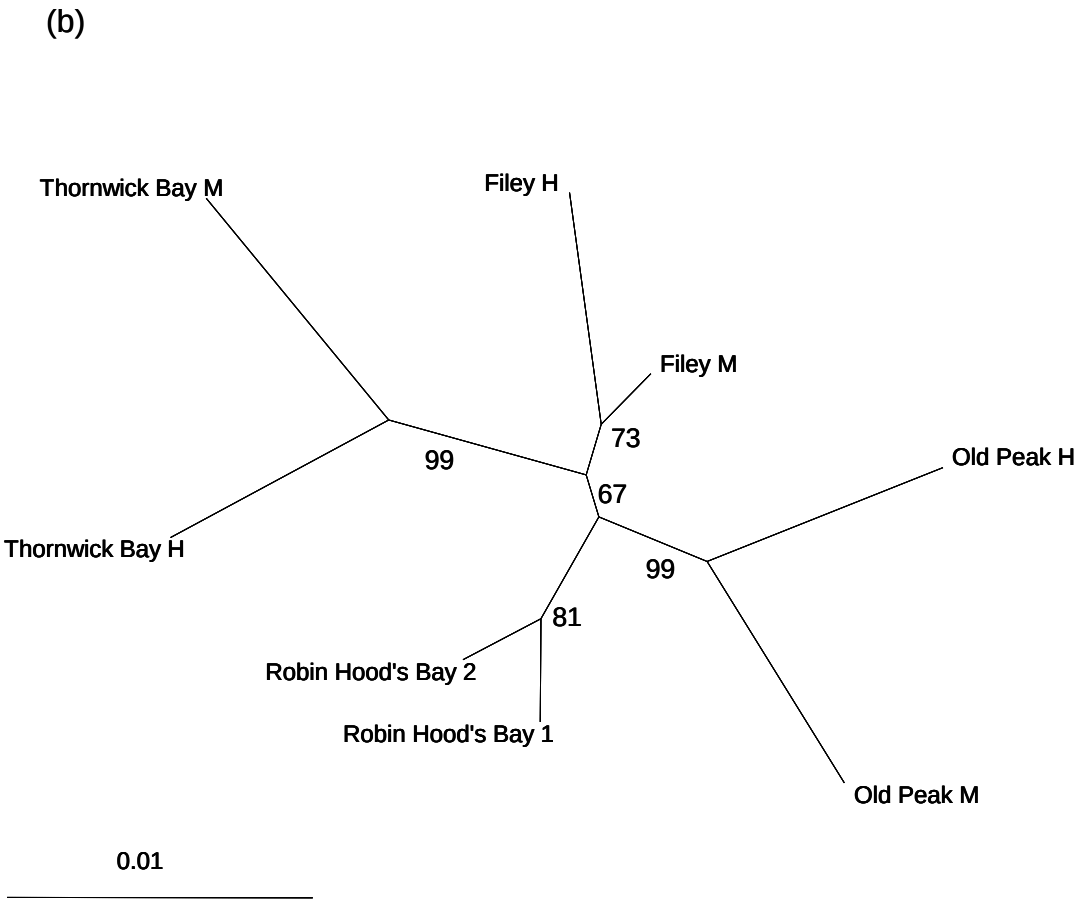

(a)
Old Peak H
Thornwick Bay H
Filey H
87
99
Robin Hood's Bay M2
90
Filey M
76
Robin Hood's Bay M1
88
Old Peak M
Thornwick Bay M
0.01

Fig. 3


(b)
Thornwick Bay M
Filey H
Filey M
73
99
Old Peak H
67
Thornwick Bay H
99
81
Robin Hood's Bay 2
Robin Hood's Bay 1
Old Peak M
0.01