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Title page

ADAPTATION TO A STEEP ENVIRONMENTAL GRADIENT AND AN ASSOCIATED BARRIER TO GENE EXCHANGE IN *LITTORINA SAXATILIS*

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Abstract.—Steep environmental gradients offer important opportunities to study the interaction between natural selection and gene flow. Allele frequency clines are expected to form at loci under selection but unlinked neutral alleles may pass easily across these clines unless a generalized barrier evolves. Here we consider the distribution of forms of the intertidal gastropod *Littorina saxatilis*, analyzing shell shape and AFLP loci on two rocky shores in Britain. On the basis of previous work, the AFLP loci were divided into differentiated and undifferentiated groups. On both shores, we have shown a sharp cline in allele frequencies between the two morphs for differentiated AFLP loci. This is coincident with a habitat transition on the shore where the two habitats (cliff and boulder field) are immediately contiguous. The allele frequency clines coincide with a cline in shell morphology. In the middle of the cline, linkage disequilibrium for the differentiated loci rises in accordance with expectation. The clines are extremely narrow relative to dispersal, probably as a result of both strong selection and habitat choice. An increase in F_{ST} for undifferentiated AFLPs between morphs, relative to within-morph comparisons, is consistent with there being a general barrier to gene flow across the contact zone. These features are consistent either with an episode of allopatric divergence followed by secondary contact or with primary, non-allopatric, divergence. Further data will be needed to distinguish between these alternatives.

Key words – adaptation, AFLP, clines, ecotone, gene flow

There has recently been renewed interest in adaptation to environmental gradients and the possibility, or perhaps the likelihood, that such adaptation may lead to speciation in the face of gene flow (Via 2002). One factor in this is the development of new models in which competition (Dieckmann and Doebeli 1999; Doebeli and Dieckmann 2003) or predation (Doebeli and Dieckmann 2000) appear to generate divergence and reproductive isolation more readily than in previous theory—but see Abrams (2001), Waxman (2004). Another factor is the use of molecular marker techniques which make hitherto intractable species into exciting new models for the study of reproductive barriers (Luikart et al. 2003).

For any particular example of clinal variation in adaptive traits on an ecological gradient, two interlocking sets of issues must be considered. One is to do with spatial distribution and history. The populations may have been isolated for a time during which they had the opportunity to evolve specialization in the absence of gene flow. Then, upon secondary contact, there may be at least a partial barrier to gene flow between the populations arising from differential adaptation, genetic incompatibility, or both. Alternatively, divergence may have occurred *in situ* in which case the barrier would be entirely due to differential adaptation since alleles causing incompatibilities are unlikely to be fixed—but see Navarro (2003). These scenarios are difficult to distinguish because similar clines in adaptive traits are expected while introgression of neutral markers can remove the signal of past separation (Barton and Hewitt 1985). In either case, isolation may increase through reinforcement (Servedio and Noor 2003) or decrease through introgression. Allopatric and sympatric phases may both contribute to the evolution of reproductive isolation as is shown by the finding that, in the fruitfly *Rhagoletis pomonella* (Walsh), the shift of populations of flies to novel

hosts involves sympatric differentiation relying on genetic variation acquired in allopatric populations (Feder et al. 2003a).

This leads to the second issue, which is the extent of the barrier to gene exchange generated by adaptation and how it comes about that groups of genes appear together persistently in the face of gene flow and recombination. How are favored gene combinations preserved and how does the barrier to gene exchange at, and near, selected loci evolve into a generalized barrier?—cf Wu (2001). An emerging answer is that groups of genes may be 'protected' from disruption through recombination by being in chromosomal rearrangements (Feder et al. 2003b; Rieseberg et al. 1995), although this has yet to be demonstrated in some well-studied model systems (Hawthorne and Via 2001; McKinnon and Rundle 2002). Further studies of differential gene exchange, and of the genomic distribution of loci that are protected from introgression, are needed to establish the relative roles of local adaptation and genetic incompatibility in generating barriers and the influence of suppressors of recombination such as chromosomal inversions in spreading their effects to the wider genome.

The intertidal snail *Littorina saxatilis* (Olivi) is a widespread species on North Atlantic coasts. *L. saxatilis* occurs in a wide diversity of habitats, from the extremely exposed oceanic island of Rockall (Moore 1977) to enclosed and sheltered lagoons and salt marshes. Reid (1996) gives an account of its distribution, shell variation and taxonomy. It is ovoviviparous, lacking a planktonic dispersal phase, and this feature suggests that it may be very prone to local adaptation. There is, as yet, no reliable estimate of lifetime dispersal distance but our observations, and those of others (Janson 1983) suggest that it is in the region of 2---100 m. Dispersal may be very dependent on population density, dispersal rates being higher at lower densities

(Johanesson and Johannesson 1995). For an intertidal species comparable to the mid-shore form of *L. saxatilis, Bembicium vittatum* Philippi, Johnson (1995) estimated lifetime dispersal to be 50---60m from direct observations and 150---300m from genetic structure.

Hull et al. (1996) showed that on the northeast coast of England there was evidence for a partial reproductive barrier between what those authors described as H and M forms of *L. saxatilis*. The H morph snails were smaller with thinner shells and wider apertures than were M, where the shells were larger, much thicker and more robust with narrower apertures. The two forms lived in different habitats: H were found higher on the shore in crevices on cliffs or very large boulders, so large as to be unlikely to be moved even by exceptional storms, while M occurred lower down the shore on smaller boulders scattered across wave-cut platforms. The authors reported that, very occasionally, they found snails with shell characters intermediate between H and M (form I). They showed that the brood characters of the two forms were different, with H females having fewer, larger eggs and embryos than did M females where the eggs and embryos were both smaller and more numerous. In each of these forms, uncleaved eggs fell into a unimodal size class distribution. A reproductive barrier was suggested because I females showed a bimodal distribution of egg size, with an unusually large proportion of aborting embryos; this initial inference was strengthened when it was shown that there was strong assortative mating between the morphs, both on and between shores (Hull 1998; Pickles and Grahame 1999). It was considered that the reproductive barrier was likely to be only partial. Subsequent work has shown that shells conforming to the H and M morphotypes occur at sites widespread in the British Isles (Wilding et al. 2002). Distinct pairs of morphs have also been described on shores in Sweden (Janson 1983) and Spain (Johannesson et al.

1995). In Spain the morphs show habitat choice and a degree of assortative mating which both contribute to a partial reproductive barrier between them (Erlandsson et al. 1999; Rolán-Alvarez et al. 1999). In Sweden there is evidence of habitat-related variation in morphology and survivorship (Janson 1983) and in enzyme polymorphism (Johannesson and Tatarenkov 1997). However in these populations there was no evidence of a partial reproductive barrier between them, although Johannesson (1997) considered that they could not yet exclude the possibility of "somewhat impeded gene flow among subpopulations of different habitats at a local scale".

The strong association between shell-shape polymorphism and habitat within *L. saxatilis* strongly suggests local adaptation in response to the very marked selective gradients that exist on rocky shores (Boulding and Hay 1993; Boulding and Van Alstyne 1993; Vermeij 1987). The heavier shell and narrower aperture of the M morph have been considered adaptations to avoid crab predation or damage by moving rocks (Raffaelli 1978) while the wide aperture and thin shell of the H morph may be optimal in the absence of these threats. Thus the two morphs in Britain appear to diverge in three ways: through different shell morphologies on an ecological gradient, by genetic incompatibility leading to embryo abortion, and by partial assortative mating, making this species a most valuable model for the study of genetic differentiation in the wild.

Wilding et al. (2001) used Amplified Fragment Length Polymorphism (AFLP) markers in British populations (principally those on the Yorkshire coast) to address the question of the genomic extent of the barrier to gene exchange between morphs. They showed that the general level of differentiation between morphs was low (mean F_{ST} ~0.04) but that there was a small group of loci (15 of 306) that showed

considerable differentiation between H and M populations on the same shore. This differentiation was consistent across three shore locations up to 45 km apart. Thus, if the populations were clustered according to genetic similarity, they grouped by morph (not geography) if the 15 differentiated loci were included in the analysis, but by geography (rather than morph) if these loci were excluded. Moreover, the behavior of these 15 loci was significantly outside the range of variation that might be expected at genetic drift – mutation – gene flow equilibrium, given a uniform rate of gene exchange across all loci. Therefore, Wilding et al. (2001) concluded that the 15 differentiated loci were likely to mark areas of the genome under selection, either for local adaptation to the shore gradient or as a result of genetic incompatibilities between morphs.

In the present paper, we build on our earlier study by analyzing AFLP and morphological variation on a fine scale within two of the sites used by Wilding et al. (2001). Specifically, we examine the form, position and width of the clines in allele frequency between morphs for the 15 differentiated loci, comparing these clines with the transition in shell form and habitat. Cline widths provide information on the strength of selection acting on these loci. We also consider patterns of spatial variation at the remaining AFLP loci. This allows us to test whether a barrier to gene exchange exists in parts of the genome that are apparently not directly influenced by loci under strong selection.

MATERIALS AND METHODS

Shores and Sampling

Habitat description

Samples were taken on two shores, Thornwick Bay (0° 07'W, 54° 08'N) and Old Peak (0° 29'W, 54° 24'N), 41 km apart on the northeast coast of England. Sample stations at Thornwick Bay are shown in Fig. 1A, distributed on the cliff wall around and above a boulder field, and in the boulder field itself. The cliff is populated by *L. saxatilis* H, the boulder field by *L. saxatilis* M. We collected snails within an area always less than 1 m^2 in the boulder field, and 0.3 m^2 on the cliff (where snails are more abundant), reflecting a compromise between sample size and area sampled. We sought to collect so that we would include snails from an H population as far as possible from influence by M (station 0, a single collection from a cliff above a rock platform on which *L. saxatilis* was absent), and also M as far as possible from influence by H (station 7, two collection sites in the boulder field). Stations 1 to 4 were positioned down the cliff from the top of the *L. saxatilis* zone to the foot of the cliff where it enters the boulder field, with station 4 situated in the boulder/cliff junction. At each of these stations, we were able to ensure that all samples were from the same vertical position, to within a few centimeters. Stations 5 and 6 were in the boulder field between the two cliff faces. At these stations (and station 7), the complex boulder habitat meant that stations, and samples within them, extended over a vertical range of 30---40cm. In the earlier study, Wilding et al. (2001) sampled in locations close to stations 1 and 6 here.

Surveying was carried out using a Leica TC400 Electronic Distomat, allowing measurements of horizontal distances and heights with respect to a point of origin in

the survey area. The vertical distances between the stations are shown as heights on Fig. 1A, arbitrarily taking the lower sample from station 7 to be 0 (although this level is just below mid-tide).

Fig. 1B shows the distribution of samples at Old Peak. On this shore the cliff itself consists of eroding, friable material, and is devoid of snails. *L. saxatilis* H are found on very large boulders below the cliff, the most extensive population being on a group of boulders from which we took six samples of H snails. M snails are found in sparsely distributed groups on much smaller boulders scattered widely over the nearly flat bedrock of the shore, from these we took nine samples of M snails. The configuration of this shore makes two important contrasts with Thornwick Bay: at Old Peak, M habitat is discontinuous from H habitat and, since the boulders here are scattered rather than piled into a boulder field, the M habitat itself is much broken up. Our sampling strategy was to find boulders which allowed reasonable numbers of snails to be collected, and we were careful in the region of large boulders with H snails to ensure that we collected H and M snails from as near to one another as they were living. Because of the nature of this shore we were unable to take replicates at stations in the pattern adopted for Thornwick Bay.

Expression of spatial data

Wilding et al. (2001) showed that when samples from populations on cliff (Thornwick Bay), or upper shore large boulders (Old Peak), were compared with samples taken from the mid-shore habitat, there was evidence of genetic differentiation between morphs (overall, but especially at a small subset of loci). In the present study, we sought to further explore variation within these habitat designations, and also between them. Therefore, a crucial estimate is the spatial distance between samples. At Thornwick Bay, the smallest distances are the heights

of the stations on the cliff for stations 1 to 4 (Fig. 1A), but for all other comparisons the changes in vertical height were small compared with horizontal distance and therefore we have used the latter. Similarly, for Old Peak spatial distances we have used the larger (in most cases, very much larger) horizontal distances rather than the small vertical ones.

Shells and Morphometrics

In the field we noted whether a sample appeared to consist at least mainly of H or M morph shells, as well as noting exactly where it was from. In the laboratory each shell from a sample was imaged digitally, after which the shell was broken for diagnosis of the snail. If this was a female *Littorina saxatilis,* it was kept for DNA extraction, otherwise both snail and shell image were discarded. Males were not included because they cannot always be reliably distinguished from *L. arcana* (Grahame and Mill 1989; Reid 1996).

Shells were measured using SigmaScan™ software, using the truss measurements originally defined by Grahame (1989) and shown in Fig. 2A. Before analysis, raw linear measurements were expressed as ratios of the geometric mean size and then transformed to base 10 logarithms, these procedures reduce the effect on the analysis of size differences as such, and normalize the data (Grahame and Mill 1989). Shape data were analyzed using Proc CANDISC and Proc DISCRIM in SAS (SAS_Institute_Inc. 1990).

Genetic analysis

AFLP methods were identical to those used by Wilding et al. (2001). Allele frequencies at putative AFLP loci were estimated from presence and absence of bands, assuming Hardy-Weinberg equilibrium. Discriminant Analysis of AFLP phenotypes was based on the original band presence-absence data rather than the estimated allele frequencies. Pairwise linkage disequilibria were calculated using Hill's formula (Hill 1974) for dominant loci. We determined the expected means and distributions of disequilibria by simulating samples with the same numbers of loci and individuals as our observed samples. For undifferentiated loci, we drew allele frequencies at random from a uniform distribution (0 to 1). For differentiated loci, we used the observed allele frequencies. We then drew AFLP presence/absence phenotypes from a binomial distribution with the mean frequency of the absence phenotype equal to the square of the absence allele frequency. One thousand simulated data sets were created for each sample and analyzed in the same way as the observed data. Simulations and analyses were conducted using Genstat7.0 (Lawes Agricultural Trust©; supplied by VSN International, Hemel Hempstead, UK).

F_{ST} was calculated using Nei's method with the Nei and Chesser correction (Nei and Chesser 1983), in order to retain comparability with the values in Wilding et al. (2001). We also estimated F_{ST} using Hickory v1.0.3 (Holsinger et al. 2002). This package uses a Bayesian approach and allowed us to test the impact of assuming Hardy-Weinberg equilibrium by comparing F_{ST} estimates (θ, Weir and Cockerham 1984) from a model in which F_{IS} is set to zero with one in which it is unconstrained. Although Hickory can estimate F_{IS} from dominant loci, the estimates are unreliable when there are many loci and small numbers of individuals per sample, as in our data.

Therefore, comparison with the model in which F_{IS} is unconstrained is more appropriate. Simple and partial Mantel tests for isolation by distance were conducted using the 'zt' package (Bonnet and Van de Peer 2002).

RESULTS

Variation in shell shapes and AFLPs

Fig. 3 shows the estimated density of female *Littorina saxatilis* at the Thornwick Bay stations, except for the first (station 0) and last (station 7) for which we did not make density estimates. Densities at these stations appeared very like those at similar levels elsewhere. The data suggest that there is a density trough where the two habitats meet: a feature we have recognized repeatedly when sampling at this site, where (unusually for this coast) the cliff habitat and the boulder field are contiguous. However, this trend is not statistically significant.

We did not make any density estimates at Old Peak. At this site, there is no feature comparable to the cliff base that marks the transition between habitats at Thornwick Bay. The complex three-dimensional form of the shores means that we could not use standard cline fitting routines, such as the methods used by Bridle et al. (2001), for example. Therefore, the most satisfactory way of comparing the spatial distributions of shell forms and AFLP genotypes at the two sites is to arrange the sample sites on a scale reflecting the distance from each sample to its nearest sample of the other morphotype (see 'Methods'). We placed the
distance-axis zero between the closest H and M samples such that H samples appear at negative and M samples at positive distances. At Thornwick Bay (Fig. 4A,B) the morphs were closest in samples taken from the foot of the cliff where it meets the boulder field (station 4, Fig. 1A). At Old Peak, the samples physically nearest one

another and considered to represent H and M morphotypes were not associated with such a clear physical feature (Fig. 4C,D).

We carried out a Canonical Discriminant analysis (Proc CANDISC in SAS) on the transformed shell data (see Methods). In trials we obtained substantially the same result whether we used the original sample as a class variable, or grouped samples into stations so that this was now the class variable. For the analysis reported here, we used station as a class variable: there was one eigenvalue >1, explaining 92% of the variation among stations (Table 1). The traits contributing most to this axis are aperture width (loading -0.58) and whorl width 1 (-0.50), with columella length (0.23) and apical angle (0.49). Fig. 4A shows the scores for individual shells on the first canonical variate plotted against the distance scale described above. There is a pronounced change in score between samples from stations 2, 3 and 4: there is a sigmoid cline centered on station 3—namely, low on the cliff habitat—with a width of approximately 2m. Clines in allele frequency for each of the 15 AFLP loci identified as differentiated (Wilding et al. 2001) are also centered on station 3, with the largest changes between stations 2 and 4. In Fig. 4B, the result of a discriminant analysis is shown, using as data the presence or absence of bands for the 15 AFLP loci. As for morphology, there was one eigenvalue >1 accounting for 91% of the variation among stations (Table 1), this high proportion reflecting the congruence in cline position and width among loci. The cline in canonical variate score for the AFLP loci is very similar, both in position and width, to the morphological cline. The Spearman rank correlation coefficient between the scores for the individual snails on canonical variate 1 from the two analyses is -0.742, $P \leq 0.0001$. Fig. 2B shows the outlines of two shells, those of snails with the highest and lowest scores on the first

canonical variate for AFLP data. This illustrates the congruence between genetic and morphological differentiation.

The Old Peak snails also suggest an abrupt transition for both shell shape and differentiated AFLP loci, in this case over a distance of approximately 4 m, although with fewer intermediates than at Thornwick Bay (Fig. 4 C,D). The Spearman rank correlation coefficient between the scores for the individual snails on canonical variate 1 from the two analyses is -0.779, $P \leq 0.0001$.

The eigenvalues show that on both shores the greatest proportion of the variation captured in the Canonical Discriminant analyses is related to H—M difference (Table 1). At Thornwick Bay this is the only variation which is significant, while at Old Peak there is evidence of an unexplained component of variation in AFLP band occurrence – but this is associated with only 10% of the overall variation, 74% being associated with the cline shown in Fig 5d. The findings reported here using Canonical Discriminant analysis (proc CANDISC in SAS) were corroborated using Discriminant Function analyses (proc DISCRIM in SAS), with geographically extreme samples as the training sets. The resulting classification of individuals from near the ends of the cline at Thornwick Bay was found to be perfect with respect to assigned morphotype or genotype, while it was less good in the middle of the cline. At Old Peak, where intermediates are less developed, classification was perfect except for two haphazardly misclassifying individuals from among the M group.

Gene flow within and between morphs

To test whether a general barrier to gene flow between H and M morphs exists, we analyzed the pattern of variation among samples for the ‘undifferentiated’ AFLP loci, i.e. all polymorphic loci other than the 15 identified by Wilding et al. (2001) as

putatively under selection, or closely linked to loci under selection. 290 of the 306 AFLP bands scored showed variation and so this analysis was based on 275 putative loci. Initially, we calculated F_{ST} values under the assumption that $F_{IS} = 0$ but this assumption was later relaxed, see below. For each site, we constructed a matrix of genetic distances ($F_{ST}/(1-F_{ST})$) (Rousset and Raymond 1997), a matrix of approximate spatial distances (horizontal distance in the boulder field plus vertical distance on the cliff, where appropriate; log scale), and a matrix identifying whether the comparison was within morph (0) or between morphs (1). Samples with intermediate mean phenotype (stations 3 and 4) were omitted. Partial Mantel tests showed that there was no significant isolation by distance at either site (when controlling for the within vs. between morph matrix) but that the genetic distance between samples of different morphs (H:M) was significantly greater than between samples of the same morph (H:H or M:M) (Fig. 5). There was weak evidence for isolation by distance within the H morph when analysed alone (Mantel coefficient = 0.34, P = 0.016, at Old Peak; 0.32, P = 0.065, at Thornwick Bay) but not within the M morph (Mantel correlation = -0.170 at Old Peak, -0.24 at Thornwick Bay; not significant in either case). This difference may indicate that mean dispersal distance is greater in the larger M morph and so samples were not sufficiently widely spaced to detect population structure. Nevertheless, the increase in F_{ST} across the contact (Table 2) clearly indicates a substantial barrier to gene flow: equivalent to a distance greater than our maximum within-morph sampling distance (on the order of 100m).

Bayesian analysis using Hickory showed that estimates of F_{ST} were higher when F_{IS} was fixed at zero (local subpopulations in Hardy-Weinberg equilibrium) than when it was free to vary (Table 3). However, whichever model was used,

differentiation between H and M was greater than differentiation among samples within morphs.

Linkage disequilibrium

Where differentiated populations exchange genes, elevated linkage disequilibrium is expected to occur. The observed disequilibrium depends on a balance between the rate of introduction of 'parental' allele combinations by dispersal, which does not vary among loci with similar cline widths, and the rate of recombination, which is specific to each pair of loci. Therefore, we tested the expectation of increased disequilibrium among differentiated loci, but not undifferentiated loci, in the centre of the H:M transition on each shore. The pattern of pairwise disequilibria among loci also potentially provides an initial insight into the genomic distribution of the differentiated loci: if all 15 differentiated AFLPs were tightly linked within an inversion, for example, we would see a uniform increase in disequilibrium across all pairwise comparisons whereas, if they were widely dispersed around the genome, we might find no detectable disequilibrium.

We estimated linkage disequilibrium for each pair of loci in each sample using the formula of Hill (1974) and compared the observed distributions with expected distributions based on the same sample sizes and allele frequency distributions in the absence of disequilibrium. The results of this analysis are shown in Fig. 6. For undifferentiated loci, positive disequilibrium means an association between presence (or absence) alleles at the two loci. For these loci, pairwise disequilibrium was concentrated close to zero in all samples, regardless of position on the shore, as expected (Fig. 6A,B,C). A small number of pairs of loci show negative values but this

pattern is also apparent in the simulated distributions. In the case of differentiated loci, 'directional disequilibrium' was given a positive sign for association between alleles typical of the same morph, negative for association between alleles typical of opposite morphs. At Thornwick Bay, average disequilibrium was low in H samples (Station 0: mean D = 0.0048, mean D/D_{max} = 0.083, exceeded by 159/1000 simulated values, Fig. 6D) and in M samples (Stations 6 & 7: mean D = 0.0026, mean D/D_{max} = 0.080, exceeded by 302/1000 simulated values, Fig. 6F) but higher in the middle of the cline, as expected (Stations 3 & 4: mean D = 0.040, mean D/D_{max} = 0.301, exceeded by 2/1000 simulated values, Fig. 6E). Amongst these loci, two pairs of bands are each separated in size by a single base pair and may be allelic (bands D19 and D20, D26 and D27). These pairs had strongly positive disequilibria in some samples but not others and removing them alters mean D only marginally (to 0.039). Comparisons between observed and expected distributions show that the shapes are similar. In the zone centre, a wider range of values is possible because of the intermediate allele frequencies and the observed mean is greater as a result of a general upward shift rather than the presence of a few unexpectedly high values (Fig. 6E). This suggests that there is no subset of tightly-linked loci. At Old Peak, where there are no samples with intermediate allele frequencies for the differentiated loci, all distributions are similar to the Thornwick Bay samples outside the contact zone (data not shown).

DISCUSSION

Wilding et al. (2001) found that populations of *Littorina saxatilis* on three shores separated by up to 45 km showed differentiation at 15 AFLP loci, out of 306 such loci identified. They suggested the existence of a cline of shell characters between H and M morphs, and that the differentiated loci were under the (perhaps indirect) influence

of selection related to the morphological differentiation between the H and M morphs and their adaptation to different sections of the steep environmental gradient down each shore. Here, we report on a finer scale analysis of two of these shores. The differentiated loci earlier identified are again found to show differentiation, with a sharp cline in allele frequency in mid-shore (Fig.4B,D). On the first of these shores, Thornwick Bay, the cline coincides with a transition in habitat from vertical cliff face to boulder field. On this shore, the two habitat types are truly contiguous: the boulders are tightly packed and press against the bottom of the cliff. A steep cline in both morphology and differentiated AFLP allele frequencies is centered on this habitat transition. At Old Peak, the habitat is not continuous, but consists of high-shore, large boulders, and mid-shore smaller boulders scattered on bedrock. Although the high-shore boulders are tumbled up against one another, the distribution of suitable crevices means that the snails are scattered in dense aggregations when sampled at low water. By careful searching, we sought to take samples of H and M snails as close to one another as was possible, and our closest distance between the two morphs was 3.75 m. There is again a steep cline between the two morphs.

On both shores, the cline estimated for the 15 differentiated AFLP loci coincides closely with a cline in shell shape (Fig. 4). This is reflected in the correlations between the scores for the individual snails on the first canonical variate in the AFLP and shape analyses (r_s = -0.742, Thornwick Bay; -0.779, Old Peak) and in the shell form of those snails identified as extreme H or extreme M on the basis of their differentiated AFLP loci (Fig. 2B). The steepness of the clines, and the congruence between those for AFLP loci and morphology, are consistent with the operation of strong selection.

At the same time, habitat choice may be expected to be occurring: it is a widespread feature of animal behavior (Jaenike and Holt 1991). In the terrestrial snail *Theba pisana* (Mueller) the animals show a considerable degree of choice of aestivation position that is related to shell form; this behaviour may act in maintaining the shell polymorphism (Hazel and Johnson 1990; Johnson 1981). For *Littorina saxatilis* in Spain, the upper-shore ridged and banded morph (RB) of *L. saxatilis* prefers patches of barnacles rather than mussels (Otero-Schmitt et al. 1997), while the aggregation patterns of snails on the shore suggested a component of habitat choice in both RB and the contrasted smooth unbanded (SU) morphs (Erlandsson et al. 1999). However Cruz et al. (2004) considered that while migration guided by habitat choice was evident in this system, it played a minor role in the re-establishment of experimentally manipulated phenotypic gradients. They suggested that habitat choice may be in the process of evolving as a result of habitat-related fitness differences of different morphs.

In our study, where there is an actual contact zone at Thornwick Bay, the cline width is of the order of 2 m. At Old Peak, there is no contact of this sort, but there are occasional intermediate animals—Fig. 4C,D, and see also Hull et al. (1996). On the basis of estimates from the literature (Janson 1983; Johnson and Black 1995), lifetime dispersal is expected to be greater than 2m per generation and this is consistent with our own observations of isolation by distance. It appears that H snails disperse over shorter distances than M snails but that the mean dispersal distance for M snails is in tens of meters (see ‘Gene flow ..’ above). This implies that there is a combination of strong selection together with habitat choice. Selection alone cannot be sufficient to maintain a cline width of less than the standard deviation of parent—offspring distances (Barton and Hewitt 1985). However, strong selection does favor the

evolution of habitat preferences (Hazel and Johnson 1990; Jaenike and Holt 1991; Rausher 1984). This non-random movement of snails is presumably toward some feature(s) of the habitat and away from others, the nature of this has yet to be explored. Such behavior would operate to produce a density trough in the portion of habitat least favored.

At Thornwick Bay, the distribution of *L. saxatilis* seems less than perfectly continuous (Fig. 3): the H morph reaches its greatest densities on the cliff some 2 m above the boulder field, while the M morph is more abundant in the boulder field from about 1 m away from the foot of the cliff. There is a trend for density to be lowest at the foot of the cliff.

Habitat choice would also substantially reduce opportunities for matings between morphs and so increase the barrier to gene exchange. The sharp clines seen at both Thornwick Bay and Old Peak (Fig. 4) reflect such a strong barrier which is expected to retard mixing even at loci unlinked to selected loci. This is borne out by the observation of increased F_{ST} across the contact zone for non-differentiated loci (Tables 2, 3).

We would expect to find increased linkage disequilibrium in intermediate samples as a result of the mixing of differentiated genotypes, and this too is observed. Those individuals that are found in the transitional habitat between the cliff and boulder field at Thornwick Bay are likely to have hybrid ancestry and this is reflected in intermediate morphology (Fig. 4A), intermediate genotypes at differentiated loci (Fig 4B) and elevated linkage disequilibrium for these loci (Fig. 6E). Some disequilibrium can be observed even between unlinked loci at the center of a narrow hybrid zone, as in *Bombina* toads (Barton and Gale 1993), where the observed mean D of 0.037 is very similar to our value at Thornwick Bay. The distribution of disequilibria provides

no evidence for grouping of the differentiated AFLP markers into tightly linked subsets, as might be seen if they were associated with regions of restricted recombination. On the contrary, it appears that the 15 differentiated regions we have detected are independent foci of selection for local adaptation.

The observations by Wilding et al. (2001) provided no information on the strength of selection causing differentiation. The evidence presented here showing narrow, congruent clines with significant disequilibrium indicates that selection must be strong, although the likelihood of habitat choice prevents us from using cline width to provide an estimate of its intensity. We postulate that this strong selection is generated by crab predation, and also by the danger of stone damage. In what seems to be a closely analogous differentiation in Swedish *L. saxatilis*, again crab activity and stone damage are considered important in selecting for different morphologies. Thicker, heavier shells (S morph) are found in sheltered habitats, while the animals from wave-exposed habitats have thinner shells with larger apertures (E morph) (Janson 1983). The two morphs showed no sign of reproductive isolation other than that due to spatial separation (Johannesson and Tatarenkov 1997). Earlier, Janson (1983) found that E, S and I (intermediate) morphs each showed best survivorship in their native habitat. There was evidence that I morphs might have an overall lower survivorship, but this was not significant. Erlandsson and Rolán-Alvarez (1998) reported random mating between Swedish E and S morphs, in contrast to assortative mating between H and M morphs in Britain (Hull 1998; Pickles and Grahame 1999). However, Hollander et al. (2005) now report evidence of assortative mating among morphs in the Swedish populations of *L. saxatilis*. We speculate that similar selective regimes operating on *L. saxatilis* in different parts of northern Europe may be having very similar outcomes.

On the coast of Galicia, Spain, *L. saxatilis* is described as being in a state of incipient speciation (Rolán-Alvarez et al. 2004), which, it is argued, is proceeding independently on several shores – thus leading the authors to refer to parallel instances of sympatric speciation. In Galicia, the morphotypes look quite unlike H and M, or E and S. The upper shore form (RB, ridged and banded) (Johannesson et al. 1993) is a relatively large animal with a sculptured shell, both features which assist in resisting crab predation (Johannesson and Tatarenkov 1997). The mid shore form (SU, smooth unbanded) is smaller and better able to escape into crevices, thus it is considered to be better adapted to withstanding the higher stress from wave crash anticipated in the mid shore (Denny 1988). In Galicia the crab predator is *Pachygrapsus marmoratus* (Fabricius), found in the upper shore. In Britain and Sweden an important predator is likely to be the green shore crab, *Carcinus maenas* (L.), which reaches the top of its vertical distribution in the lower intertidal. The thick-shelled, relatively large M animals are likely to be adapted for resisting crab predation (or stone damage, (Raffaelli 1978), while the thin-shelled H population lives above the reach of *Carcinus*.

Schilthuizen (2000) suggested that ecotones may be "speciation-prone" – that adaptation to habitat across a pronounced environmental gradient may lead to the evolution of assortative mating, and to parapatric speciation. We consider it premature to claim the *Littorina saxatilis* clines described here, or in Galicia (Rolán-Alvarez et al. 2004) as examples of 'incipient' non-allopatric speciation for several reasons. Our data are consistent with models of introgression following secondary contact. It is difficult to exclude this possibility for the Swedish or Galician clines either. In the British case, the genetic incompatibility in the form of embryo abortion is evidence favouring a period of allopatry. Such incompatibilities could evolve in parapatry only

in regions of restricted recombination, for example in chromosomal inversions (Navarro and Barton 2003). Our data suggest that the differentiated AFLP loci are unlikely to be contained within such inversions. However, the possibility that they are physically grouped in the genome should be pursued by investigation of the genomic regions marked by differentiated AFLPs, and by cytogenetic analysis, as well as through the patterns of disequilibrium. Secondly, there is no reason to suppose that future evolution will result in complete reproductive isolation: the present interaction may be stable. Selection for reinforcement of prezygotic isolation is likely to be weak if habitat preferences result in limited opportunity for hybrid matings. Finally, we doubt whether interactions on different shores are truly independent and so provide examples of parallel evolution. Mean dispersal distances of tens of meters seem likely for the M morph at least and some individuals are probably moved much further, especially during storms. Wilding et al. (2001) detected isolation by distance over tens of kilometers, supporting the possibility of occasional long-distance dispersal. Over a span of centuries it seems highly likely that occasional dispersal between shores would be sufficient to allow the spread of favorable mutations.

We anticipate that a definitive test for parallel, independent origins of similar patterns of differentiation will come from investigation of the comparative genomics of the H and M morphs on British shores and the RB and SU morphs in Spain. Comparisons of sequence variation at or near loci crucial for adaptation to the predation-exposure gradient with data for neutral loci will make it possible to distinguish allopatric from parapatric divergence and to test the independence of different instances of divergence. For the moment, the clearest case for independent parallel divergence is on the large scale, between Sweden, Britain and Spain, and the case for sympatric differentiation within regions remains unproven.

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Table 1. Values, and percentages of among-station variation explained, for the first three eigenvalues in canonical discriminant analyses of shell shape variables and the 15 differentiated AFLP bands at Thornwick Bay and Old Peak.

	Eigenvalue 1	Eigenvalue 2	Eigenvalue 3
Thornwick Bay (shell shape)	4.02 (91.5%)	0.16 (3.6%)	0.11 (2.6%)
Thornwick Bay (AFLP bands)	5.03 (91.0 %)	0.19 (3.5%)	0.11 (1.9%)
Old Peak (shell shape)	7.87 (82.9%)	0.65 (6.8%)	0.41 (4.3%)
Old Peak (AFLP bands)	9.8 (74.0%)	1.35 (10.2%)	0.51 (3.9%)

Table 2. Mean (among comparison standard error) of F_{ST} among samples for non-differentiated loci

	Thornwick Bay	Old Peak
Within H morph	0.033 (0.0021)	0.030 (0.0024)
Within M morph	0.041 (0.0021)	0.050 (0.0033)
Between morphs	0.058 (0.0015)	0.062 (0.0025)

Table 3. Estimates of F_{ST} for non-differentiated loci using the Bayesian approach in Hickory (2.5 and 97.5 percentiles of posterior distribution)

	Thornwick Bay		Old Peak	
	F_{IS} free model	F_{IS} = 0 model	F_{IS} free model	F_{IS} = 0 model
Within H	0.0205 (0.0141-0.0280)	0.0086 (0.0050-0.0154)	0.0242 (0.0164-0.0345)	0.0138 (0.0072-0.0205)
Within M	0.0100 (0.0052-0.0157)	0.0092 (0.0049-0.0149)	0.0125 (0.0054-0.0230)	0.0073 (0.0025-0.0138)
H vs M	0.0468 (0.0336-0.0639)	0.0293 (0.0217-0.0391)	0.0585 (0.0422-0.0799	0.0390 (0.0291-0.0515)

Figure legends

Fig. 1 Sampling stations on the two shores. At Thornwick Bay (A) there were seven stations at different vertical positions on the shore (bold numbers) with up to three samples per station (letters). The heights (m) above the arbitrary reference of the lowest sample are shown, the height of station 0 is an estimate since it was out of line of sight of the survey origin (see text). At Old Peak (B), sample locations are numbered in order of greatest distance from the nearest population of the opposite morph. Open pentagons, H samples; solid squares, M samples. Vertical distances here are smaller than at Thornwick Bay, all samples being within 2 m vertical range.

Fig. 2. Outline of shell showing measurement trusses (A); the two shells identified in a Canonical Discriminant analysis for Thornwick Bay AFLP data (see text) which lie at opposite ends of the first discriminant axis (B).

Fig. 3. Estimates of density of snails at Thornwick Bay. X axis is distance along the shore, expressed as used for Figs 4A,B (see text). The transition from cliff to boulder habitat is indicated by the arrow. Densities were not estimated at the extremes, there are therefore fewer data plotted on this figure.

Fig. 4. The scores for individual snails plotted on the first canonical variate from the analysis of shell shape (A) and 15 differentiated AFLP bands (B) at Thornwick Bay, and at Old Peak, C and D respectively, in relation to their distance from the nearest sample of the opposite morph (see text).

Fig. 5 Relationship between genetic and spatial distances at Thornwick Bay (A) and Old Peak (B).

Fig. 6. Linkage disequilibrium estimates for populations at Thornwick Bay, calculated from undifferentiated loci (A,B,C) and differentiated loci (D,E,F) for H

(A,D), center (B,E) and M (C,F) samples. Bars—observed values. Points—means of simulated values from 1000 replicates of 105 pairwise comparisons (error bars give 2.5 and 97.5 percentiles) for differentiated loci or 10 replicates of 32,385 pairwise comparisons (error bars give ranges) for undifferentiated loci.

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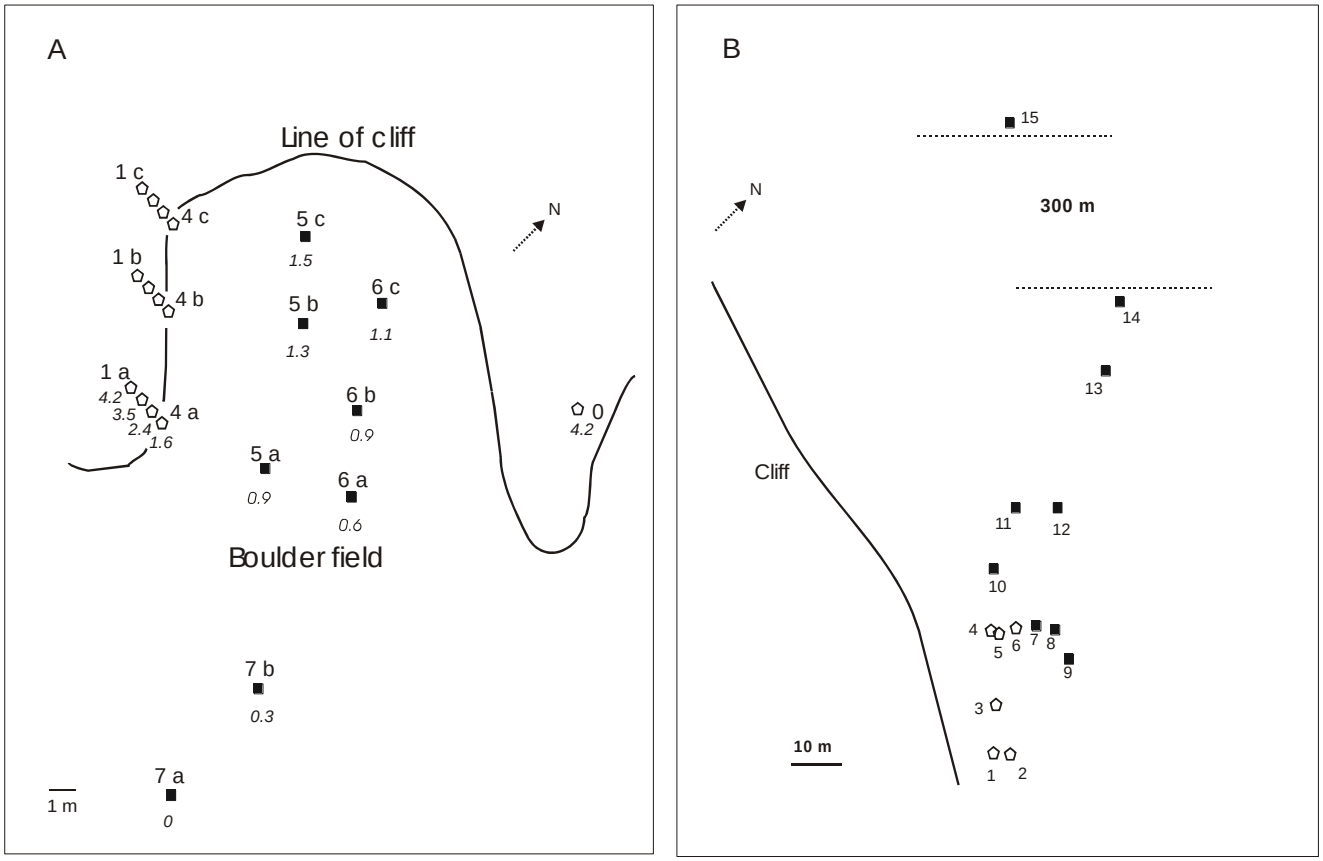

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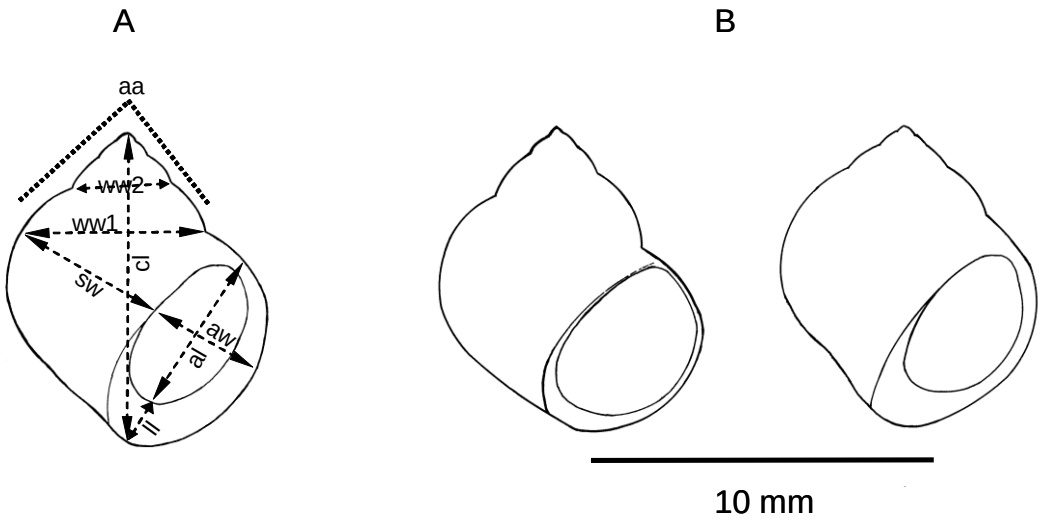

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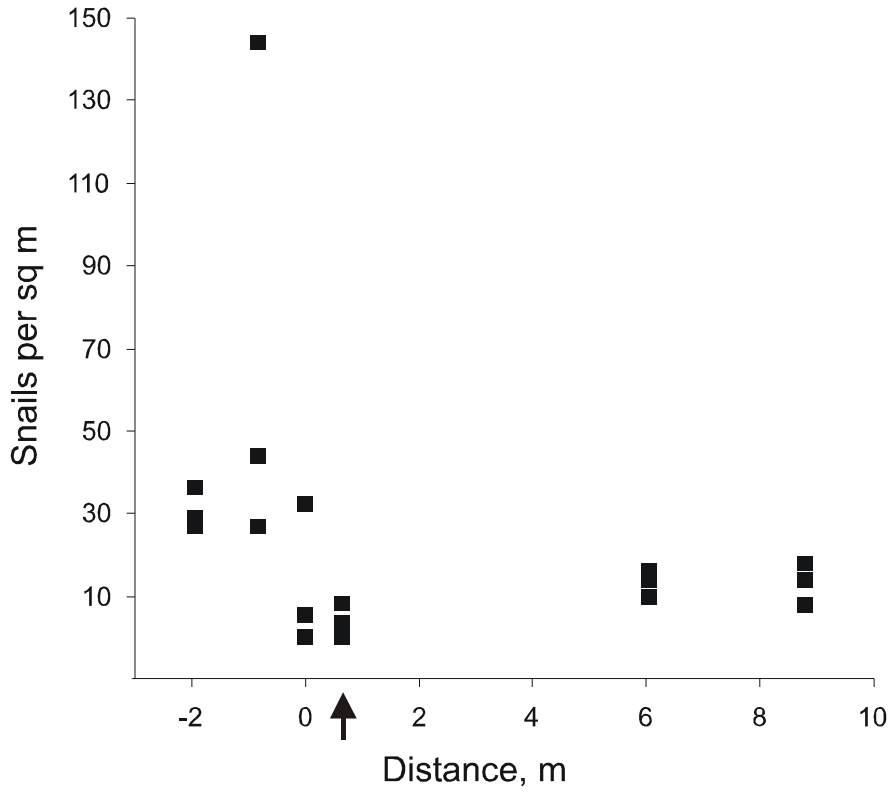

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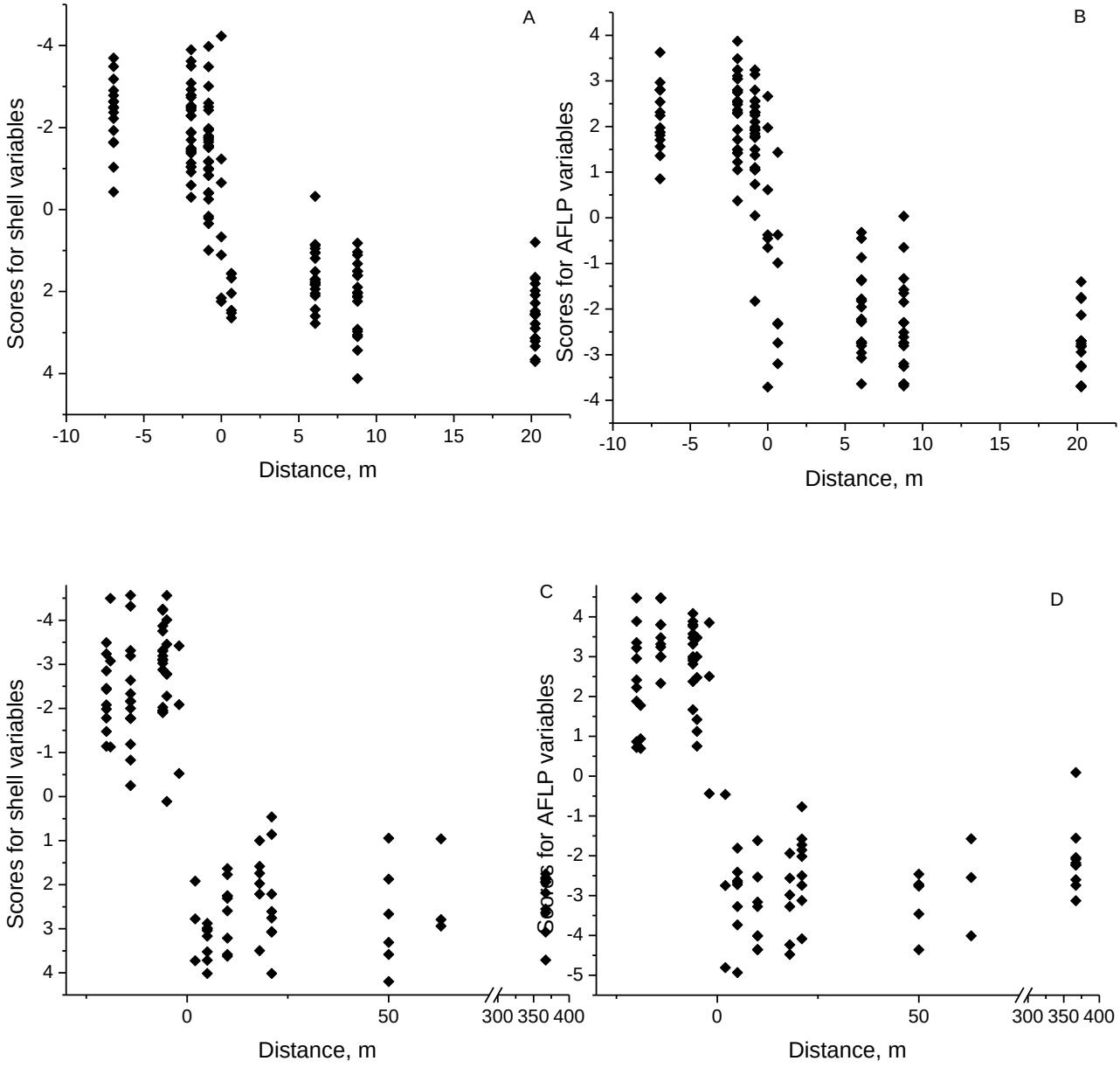

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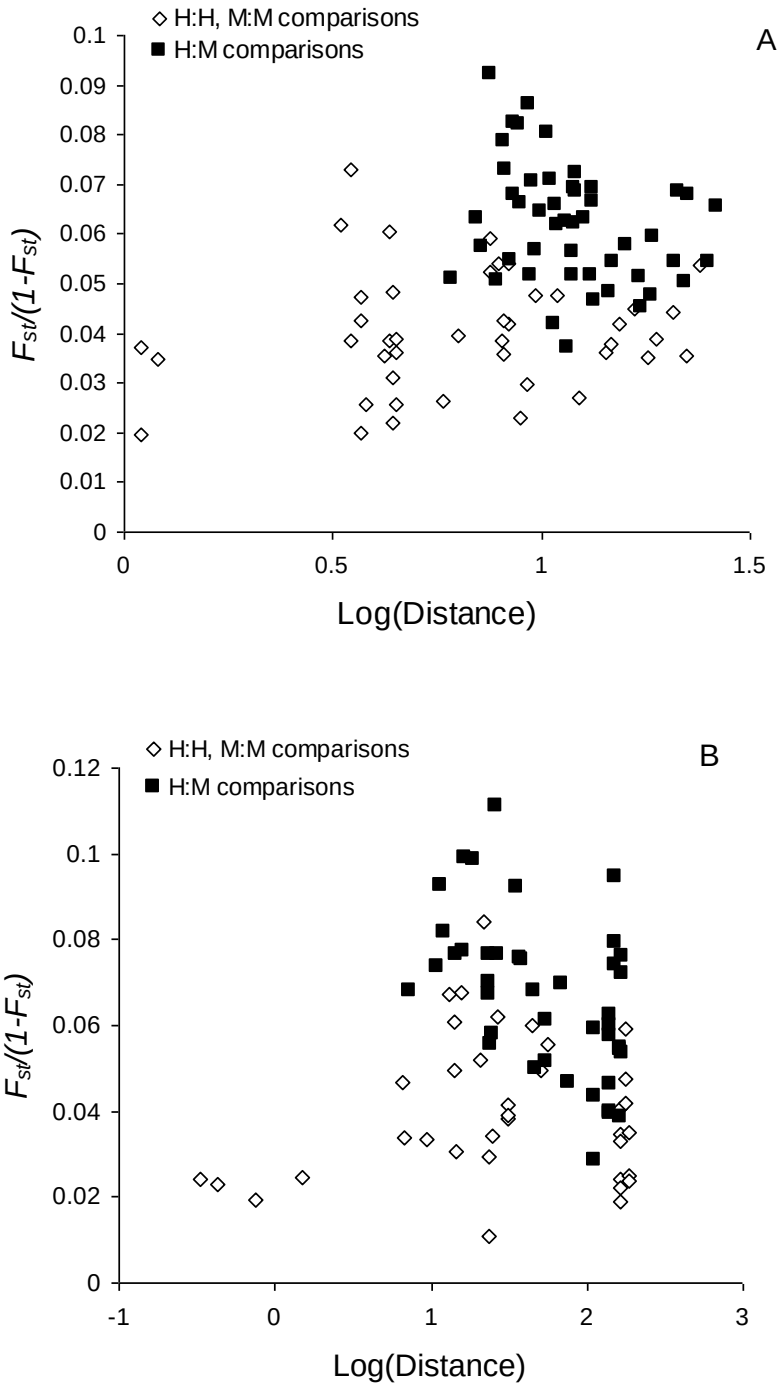

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