Genetic discontinuity associated with an environmentally induced barrier to gene exchange in the marine snail *Littorina saxatilis*

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ABSTRACT: The geographical genetic structure of 7 populations of the intertidal marine snail *Littorina saxatilis* with limited dispersal was studied in NW Spain using 3 classes of genetic markers: 840 amplified fragment length polymorphism (AFLP) loci, 3 microsatellites, and 1 morphological trait (shell size). A sharp genetic division at Cape Finisterre, as evidenced by all 3 markers, delimits 2 regions. This genetic discontinuity coincides with an abrupt change in plankton composition, climate and oceanographic characteristics, suggesting that contemporary influences on gene flow associated with the presence of an ecological barrier maintain the genetic subdivision in NW Spain. However, the genetic structure also accords to some extent with an isolation-by-distance model despite the heterogeneous gene flow due to this ecological barrier. Microsatellites and shell size showed a strong and significant covariation in pairwise differences (F_{ST} and Q_{ST}) among populations, but no correlation was found between these markers and AFLPs. Similarly, both microsatellites and shell size showed a higher differentiation between the 2 regions than within each region, but this pattern was reversed for AFLPs. Differences in genomic sampling error and homoplasy could account for the discrepancy between different kinds of markers. These results point to the possibility that other marine species may show a similar pattern of intra-specific genetic divergence in NW Spain.

KEY WORDS: Microsatellites · AFLPs · Quantitative traits · Gene flow · Q_{ST} · F_{ST} · Isolation by distance · Ecological barrier

INTRODUCTION

Understanding the processes by which populations become genetically distinct and adapt to their local environments is a central topic in evolutionary biology (Lenormand 2002). Theoretical studies show that genetic drift and local adaptation are counteracted by the homogenizing effects of gene flow (Slatkin 1987). Consequently, species with low dispersal capabilities are more likely to experience local adaptation and genetic divergence due to natural selection than species characterized by large dispersal rates (Sotka 2005). The interpretation of patterns of genetic differentiation in terms of local adaptation, however, can be difficult because demographic events, such as population fragmentation, range expansions and founder events, can mimic the effect of natural selection. Nevertheless, demographic processes are expected to affect all loci in a roughly equal fashion, whereas selection will affect only some regions of the genome. An additional difficulty in the marine environment is that barriers to gene flow are hard to discern and rarely absolute. Oceanographic features, such as biogeographic boundaries and patterns of coastal or estuarine circulation, are some of the processes by which gene flow could be limited. However, the role of barriers as a mechanism for partitioning genetic variation in the marine environment remains insufficiently understood (Avise 2004).

Natural populations of the marine snail *Littorina saxatilis* provide an excellent model system to assess the interplay between gene flow, genetic drift and

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local adaptation. This snail lives in the intertidal zone along the Atlantic shores of Western Europe and North America, is ovoviviparous, feeds on the alga microflora and shows a low dispersal capability (1 to 3 m mo^{-1}) that typically results in a pattern of isolation-by-distance (Reid 1996). The wide geographic range of this species, coupled with apparently limited dispersal opportunities, has led to a remarkable geographic and microgeographic differentiation (Reid 1996). Indeed, the species is very polymorphic in shell traits both within and between populations and shows strong population structure for allozymes, microsatellites and mtDNA (Ward et al. 1986, Panova et al. 2006, Quesada et al. 2007). Several studies demonstrate a link between ecological adaptation, vertical and/or horizontal distribution, and microhabitat phenotypic differentiation (Rolán-Alvarez et al. 2004, Grahame et al. 2006, Quesada et al. 2007). In many areas, morphologically distinct ecotypes coexist in sympatry or allopatry separated by only a few meters, each showing a significantly higher fitness in its own microhabitat (Rolán-Alvarez et al. 1997).

The NW coast of Spain (Fig. 1a) is characterized by fjord-like estuaries that represent discrete habitats isolated from each other by barriers to dispersal and environmental tolerance (Ríos et al. 1992) and constitute a singular system to assess the importance of hydrographic factors on genetic differentiation and adaptation. Interestingly, NW Spain also appears to contain at least 2 distinct biogeographic regions (to the north and south of Cape Finisterre, Fig. 1b,c) with differences in primary production, salinity, water temperature and evaporation quantities (Fraga et al. 1982, Martínez & Pérez 1999). During summer, a cold and nutrient-rich coastal current flows southward in the north (ACNAp, whereas a warmer and nutrient-poor current flows in the south (ACNAs) (Fraga et al. 1982, Alvarez-Salgado et al. 2003; see Fig. 1b). In September and October, the surface circulation reverses. One of the most characteristic features of the NW Spain hydrographic system is a quasi-permanent upwelling in summer in the area of Cape Finisterre which is due to the presence of a subsurface front between central water of distinct origins (ACNAp and ACNAs) (Fraga
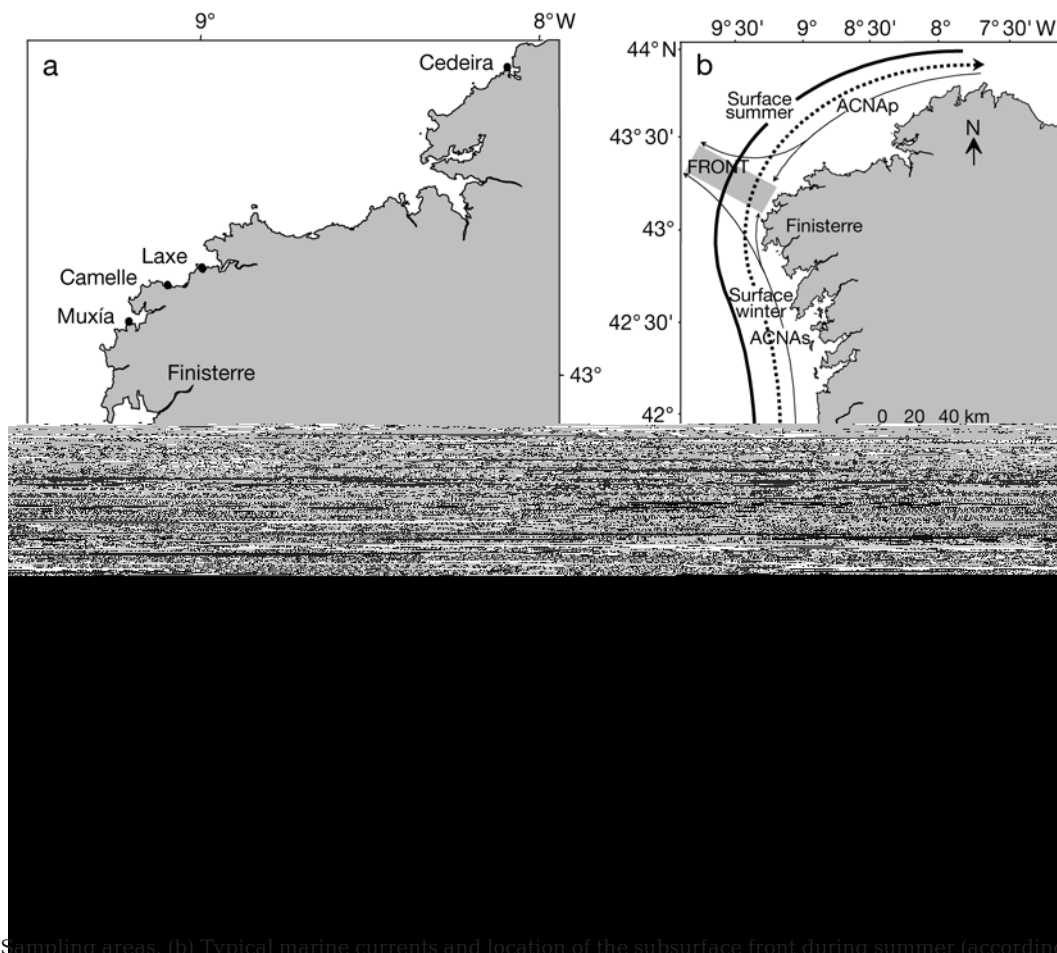

Fig. 1. (a) Sampling areas. (b) Typical marine currents and location of the subsurface front during summer (according to Fraga et al. 1982). ACNap, ACNAs: currents; see 'Introduction' for description. (c) Distribution of coastal evaporation (Martínez & Pérez 1999) and of marine gradients in temperature and salinity (Alvarez-Salgado et al. 2003)

et al. 1982). Although all these factors point to the importance of this biogeographic boundary for genetic differentiation, no study has yet considered its impact on patterns of population structure for any marine species.

In the present study we assessed the variation of 3 different markers (amplified fragment length polymorphisms [AFLPs], microsatellites and shell size) in *Littorina saxatilis* populations from NW Spain. We tested whether interpopulation genetic differentiation is gradual, as expected from the outcome of simple population genetic processes such as isolation-by-distance, or whether this genetic differentiation is discontinuous and linked to a gene flow constraint and/or adaptive processes associated with the biogeographic boundary.

MATERIALS AND METHODS

Sampling. Samples were collected June through July 2004 from 7 geographical areas of the western coast of Galicia (Fig. 1). In each area, we collected individuals from 2 sheltered localities 0.5 to 2 km apart (and 2 replicates per locality 10 to 20 m apart) to adequately represent the genetic variation of each area as a whole (20 snails per sampling site, 80 in total per area). We also extracted all developed embryos (shell height >0.5 mm) from 20 females randomly chosen from each sampling site. Four embryos per female were then chosen at random for subsequent analysis (80 embryos per sampling site, 320 in total per area). The individuals collected within a particular area were pooled in the subsequent statistical analyses.

Morphometric analysis. Shell size was measured by the centroid size, which is the square root of the sum of squared distances of landmarks (morphometric coordinate points) to their centroid (Zelditch et al. 2004). Sampled specimens were examined using a Leica MZ12 stereoscopic microscope. Color images were captured by a Leica digital ICA video camera. Embryo shell images (N = 2240) were analyzed using 12 landmarks (coordinate points) positioned on the digitalized shell image (see Fig. 1 in Carvajal-Rodríguez et al. 2005). Landmarks were recorded for each specimen with the program TPS-DIG, while centroid size was computed with TPSRELW (http://life.bio.sunysb.edu/morph/index.html).

Molecular analysis. The shells of the adult specimens of *Littorina saxatilis* (N = 560) were broken, and the head–foot tissue was used for extracting total genomic DNA, according to a standard small-scale procedure (Wilding et al. 2001).

We assessed variation at 3 polymorphic *Littorina saxatilis* microsatellite loci: *Lsax6CAA*, *Lx-12* and *Lx-18* (Sokolov et al. 2002). PCR primers and amplification con-

ditions of these 3 loci were as described previously (Sokolov et al. 2002), except that we used annealing temperatures of 61°C for *Lsax6CAA* and 60°C for *Lx-12* and *Lx-18*. The reverse primer was labeled with one of the fluorescent phosphoramidite dyes: 6-FAM (*Lsax6CAA*), HEX (*Lx-12*) or NED (*Lx-18*). The fluorescent-labeled PCR products were size-separated on an ABI PRISM 310 DNA automatic sequencer. All genotyping was performed using the software GENOTYPER 2.1 (Applied Biosystems). Scoring errors, large allele dropout and null alleles were checked employing the program MICROCHECKER (Van Oosterhout et al. 2006). GENEPOP 3.0 (Raymond & Rousset 1995) was used to determine allele frequencies and to test the significance of departures from Hardy-Weinberg equilibrium using a Markov Chain procedure. p-values were adjusted by application of a sequential Bonferroni correction.

AFLP analysis was performed using a modified version of the procedure described by Vos et al. (1995). Briefly, the genomic DNA was double digested with *Eco*RI and *Mse*I restriction enzymes. The DNA fragments were ligated with *Eco*RI and *Mse*I adaptors and pre-amplified with primers carrying 1 selective nucleotide (5′-GACT-GCGTACCAATTC+A-3′ for *Eco*RI adapter and 5′-GATGAGTCCTGAGTAA+C-3′ for *Mse*I adapter). The

We carried out a 2-level hierarchical partition of genetic differentiation (biogeographic regions and areas within regions) using an analysis of molecular variance (AMOVA) approach as implemented in ARLEQUIN (Schneider et al. 2000). For this analysis, the data were rearranged in different ways to analyze different pieces of information. At a macrogeographical level, R estimates the genetic differentiation among biogeographic regions (north and south of Finisterre) after grouping areas within regions, whereas $A(R)$ estimates the genetic differentiation among areas within regions (after grouping sampling sites within each area). The statistical significance of the genetic differentiation within each partition was determined via 10 000 permutations using ARLEQUIN.

Isolation-by-distance over the distribution area was assessed using the correlation of $F_{ST}/(1 - F_{ST})$ or $Q_{ST}/(1 - Q_{ST})$ against the logarithm of geographical distance. This model was evaluated using Mantel's test as implemented in the program ZT (Bonnet & van der Peer 2002) via 10 000 randomizations. Mantel tests were also performed to assess the correlation between pairwise F_{ST} and Q_{ST} estimates among samples.

We used nonmetric multidimensional scaling (MDS) (Kruskal & Wish 1978) to visualize patterns in the relationships among the samples in terms of the F_{ST} and Q_{ST} statistics. The relative distances among samples in these ordination plots indicate the relative genetic differentiation among *Littorina* populations, with plots having a stress value less than 0.20 providing interpretable information concerning interpopulation relationships (e.g. Clarke 1993). The statistical significance of the differences among clusters identified by the MDS analysis was assessed *a posteriori* using a randomized ANOVA test (Peres-Neto & Olden 2001). For this analysis, the coordinates on Dimension 1 from each separate cluster were randomized 10 000 times, and the probability of getting an F-value larger than

the sampled F was estimated using the program ANOVA (available at: www.webs.uvigo.es/c03/webc 03/XENETICA/XB2/software.htm).

Neighbor-joining clustering was performed with PHYLIP (Felsenstein 2004). Nei's genetic distance (Nei 1972) was used for microsatellite and AFLP data, whereas Q_{ST} was used for shell size. The level of support of the nodes for microsatellite and AFLP data was assessed by 1000 bootstrap replicates.

RESULTS

A total of 560 individuals, representing all 7 geographical areas, were scored successfully for the 3 microsatellite and 840 AFLP loci assayed in the present study. Allele frequencies at each locus for each population are available from the authors of the current study on request. Heterozygosity levels were higher for microsatellites (mean heterozygosity = 0.837) than for AFLPs (mean heterozygosity = 0.313), as expected from the greater allele numbers in microsatellites. Levels of genetic variability were similar across all areas for microsatellites and AFLPs, although Cedeira exhibited a slightly higher number of alleles for microsatellites (Table 1). Tests for Hardy-Weinberg equilibrium showed a consistent trend for a deficiency of heterozygotes in microsatellite loci after sequential Bonferroni correction (Table 1). Results did not change qualitatively when considering unpooled samples for each area (Table 2) or after correcting the microsatellite data for the presence of null alleles or allele dropouts. Hence, the original microsatellite data were used. Separate estimates of AFLP genetic diversity were obtained assuming both Hardy-Weinberg equilibrium and a deviation from equilibrium equal to the average deficit of heterozygotes observed in the microsatellite loci (F_{IS} = 0.245). Both methods gave roughly similar AFLP diversity estimates (Table 1).

Table 1. Microsatellite and amplified fragment length polymorphism (AFLP) variation in each *Littorina saxatilis* geographical area. H_e: Nei's unbiased genetic diversity (expected heterozygosity); na: number of alleles; F_{IS}: deviations from Hardy-Weinberg equilibrium; PL: proportion of polymorphic loci. *Significant deviation from the Hardy-Weinberg expectations after sequential Bonferroni correction

| Location | Microsatellites | | | | | | | | | AFLPs | | | |
| | Lsax6 | | | Lx-12 | | | Lx-18 | | | $F_{IS} = 0$ | | $F_{IS} = 0.245$ | |
	H_e	na	F_{IS}	H_e	na	F_{IS}	H_e	na	F_{IS}	H_e	PL	H_e	PL
Cedeira	0.837	21	0.228*	0.942	32	0.220*	0.872	25	0.173*	0.324	93.25	0.326	93.25
Laxe	0.937	19	0.329*	0.958	30	0.195*	0.609	11	0.296*	0.308	88.97	0.312	91.9
Camelle	0.936	17	0.690*	0.908	30	0.238*	0.891	19	0.236*	0.311	91.62	0.312	91.6
Muxía	0.852	12	0.134*	0.944	29	0.099*	0.709	8	0.277*	0.324	93.57	0.322	93.57
Aguiño	0.906	18	0.296*	0.597	24	0.026*	0.718	12	0.425*	0.320	93.87	0.321	93.87
Ons	0.882	16	0.192*	0.770	28	0.237*	0.830	11	0.126*	0.290	78.12	0.286	88.00
Mougás	0.864	18	0.738*	0.894	28	0.009*	0.730	8	0.041*	0.314	90.72	0.312	93.12

Table 2. *Littorina saxatilis.* Weir and Cockerham estimates of deviations from Hardy-Weinberg equilibrium per locality for microsatellite loci. H_e: Nei's unbiased genetic diversity (expected heterozygosity); H_o: observed heterozygosity; F_{IS}: deviations from Hardy-Weinberg equilibrium. *Significant deviation from Hardy-Weinberg expectations after sequential Bonferroni correction

Location	Lsax6			Lsax-12			Lx-18		
	H_e	H_o	F_{IS}	H_e	H_o	F_{IS}	H_e	H_o	F_{IS}
Cedeira1	35.545	32	0.100*	32.794	33	−0.006	27.633	26	0.059
Cedeira2	22.288	18	0.195*	32.000	24	0.252*	35.870	31	0.137*
Laxe1	23.941	21	0.125	27.135	30	−0.107	18.489	21	−0.139*
Laxe2	25.618	22	0.143	33.087	22	0.338*	17.661	8	0.550*
Camelle1	16.000	12	0.255*	26.523	30	−0.133	29.985	31	−0.034*
Camelle2	5.272	5	0.056	31.661	22	0.308*	27.355	19	0.309*
Muxia1	31.146	32	−0.027	37.708	35	0.072	24.873	36	−0.456*
Muxia2	27.910	27	0.033	37.354	33	0.117*	9.842	5	0.496*
Aguino1	30.046	30	0.001	21.683	24	−0.108	24.377	31	−0.277*
Aguino2	25.508	21	0.179*	25.987	25	0.038	12.790	2	0.846*
Ons1	31.115	30	0.036	16.278	16	0.017	30.808	33	−0.072*
Ons2	26.507	26	0.019*	29.857	28	0.063	27.813	25	0.102*
Mougás1	15.424	12	0.227*	35.708	38	−0.065	30.189	28	0.073*
Mougás2	9.190	4	0.576*	35.886	33	0.081*	27.417	28	−0.021*

The MDS of pairwise F_{ST} and Q_{ST} estimates for the 7 geographical areas generated plots with good fit to the observed data (Fig. 2). The stress values (*s*) were small for microsatellites ($s = 0.0006$), AFLPs ($s = 0.0062$) and shell size ($s = 0.0002$), where $s = 0$ indicates a perfect fit. For all 3 data sets, the MDS revealed 2 clusters that showed a clear concordance with biogeography. Differences between areas to the north and south of Cape Finisterre, grouped in separate clusters, were statistically significant ($p < 0.05$) for each data set as determined by a randomized ANOVA test comparing the Dimension 1 coordinates from each cluster. The
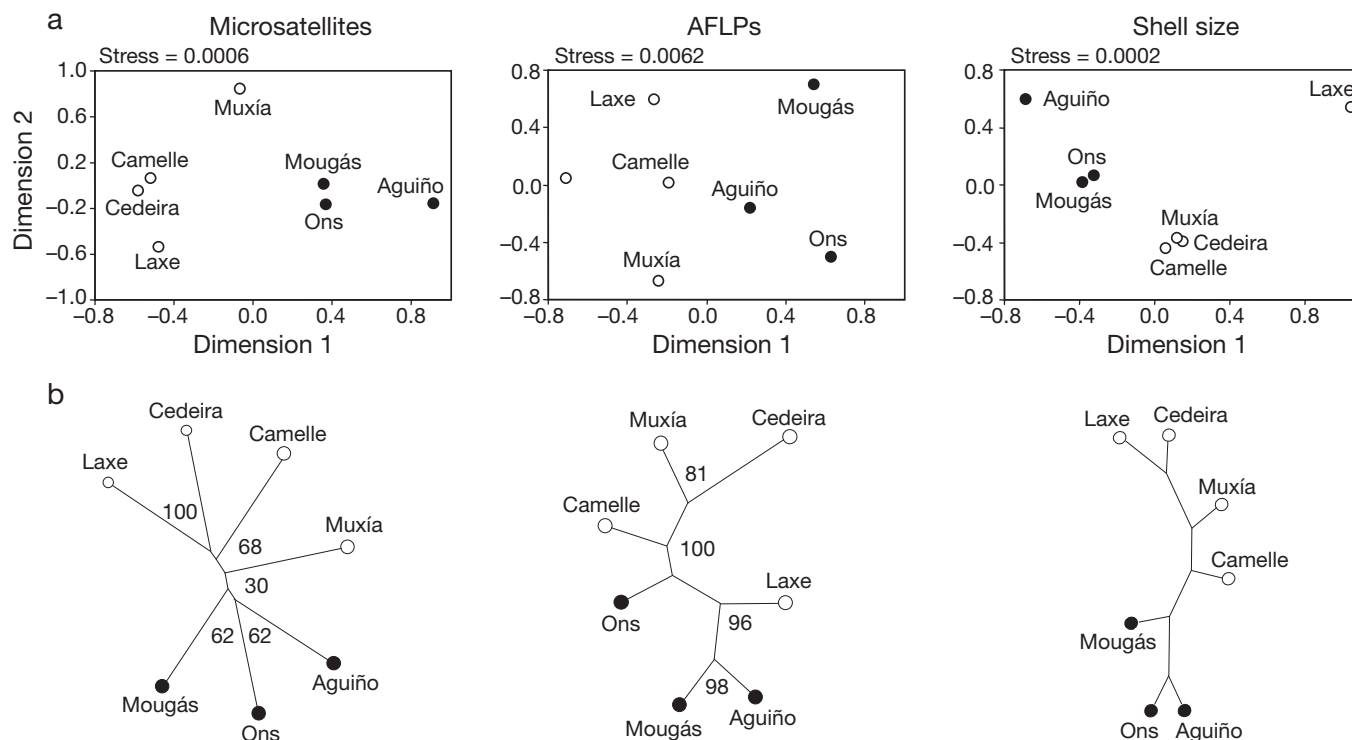

Fig. 2. *Littorina saxatilis.* Relationships among samples for each type of genetic marker. (a) Multidimensional scaling plots based on F_{ST} (microsatellites and amplified fragment length polymorphisms [AFLPs]) and Q_{ST} (shell size) estimates. (b) Neighbor-joining clustering based on Nei's genetic distances (microsatellites and AFLPs) and Q_{ST} (shell size) estimates. Bootstrap values shown at the nodes. Southern (●) and northern (○) areas
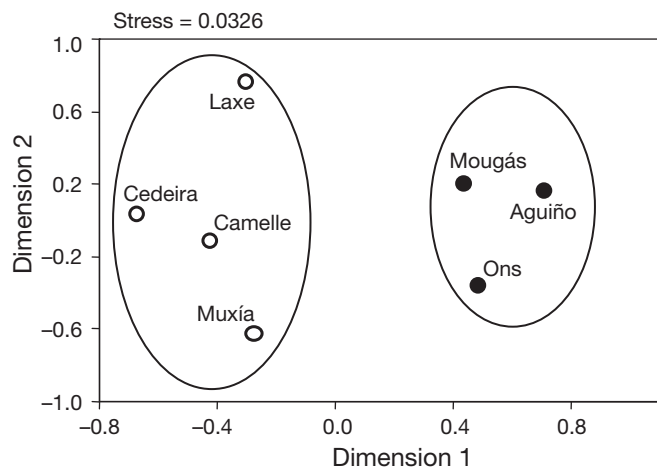
Fig. 3. *Littorina saxatilis*. Multidimensional scaling plot illustrating the relationships among samples combining the pairwise population differentiation estimates for microsatellites, amplified fragment length polymorphisms (AFLPs) and shell size. Southern populations (●) and northern (○) populations

neighbor-joining trees (Fig. 2) revealed essentially the same pattern as the MDS analyses, although Ons and Laxe showed a less evident grouping by biogreographical region in the AFLP data. The discrimination between samples from each biogeographic region was substantially increased when the multidimensional scaling combined the F_{ST} and Q_{ST} matrices for the 3 data sets (Fig. 3, s = 0.0326), resulting in highly
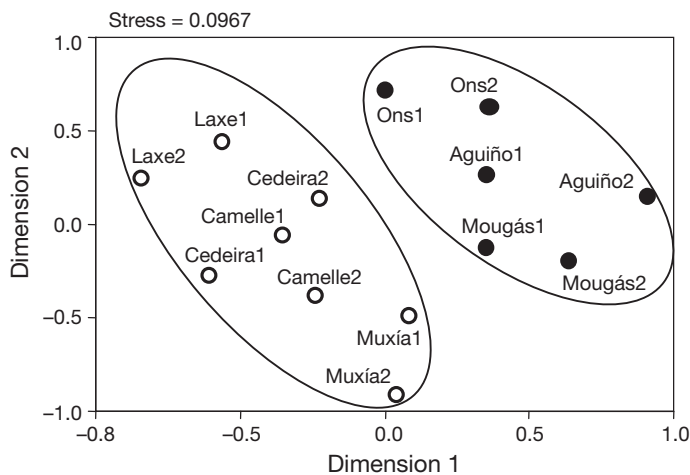
Fig. 4. *Littorina saxatilis*. Multidimensional scaling plot of the relationships among the localities sampled in each study area. Pairwise population differentiation estimates for microsatellites, amplified fragment length polymorphisms (AFLPs) and shell size were combined. Southern populations (●) and northern populations (○). Differences between clusters are statistically significant (p = 0.0015) as determined by a randomized ANOVA test comparing the Dimension 1 coordinates of each cluster

significant (p = 0.018) differences among clusters. A similar pattern was observed when the analyses were based on unpooled samples from each area (Fig. 4).

The hierarchical analysis of the distribution of genetic variation (AMOVA) indicated that variability is significantly partitioned among areas and biogeographic regions for the 3 data sets (Table 3). Microsatellites and shell size gave similar results: generally higher differences between biogeographic regions than among areas within regions, and a lower differentiation for southern than for northern populations. However, for AFLPs the between-region variance component was at least 50% smaller than the among-area component within each region, indicating that the within-region component accounts for most of the genetic diversity. Taken overall, these results indicate that AFLPs appear to have a moderate differentiation among biogeographic regions with respect to microsatellites and shell size, perhaps as a result of a differential response to local processes within regions. Consistent with this, Mantel tests among pairwise F_{ST} and Q_{ST} estimates (Fig. 5) revealed a strong and significant correlation among microsatellites and shell size for unpooled samples (r = 0.313, p < 0.05) but not between AFLPs and microsatellites (r = 0.176, p > 0.05) or between AFLPs and shell size (r = 0.097, p > 0.05). These results imply the AFLPs as the source of the lack of correlation.

Although the above results clearly indicate a genetic break associated with a biogeographic boundary, we could nevertheless identify an isolation-by-distance pattern. A scatterplot of the logarithm of geographical distance versus $F_{ST}/(1 - F_{ST})$ or $Q_{ST}/(1 - Q_{ST})$ illustrates an overall trend of increasing differentiation with increased geographical distance among unpooled samples for all 3 markers (Fig. 6). The results were highly significant for microsatellites (r = 0.267, p < 0.01) and AFLPs (r = 0.470, p < 0.001), whereas the trend was less clear and marginally significant for shell size (r = 0.147, p < 0.10). No significant results were obtained when the tests were performed separately within northern and southern populations, which, however, showed similar trends to those observed for the total data set (not shown).

Table 3. *Littorina saxatilis*. Two-level hierarchical partition of genetic variability. *R*: genetic differentiation between biogeographical regions; *A(R)*: genetic differentiation among areas within regions. AFLPs: amplified fragment length polymorphisms. *** p < 0.001

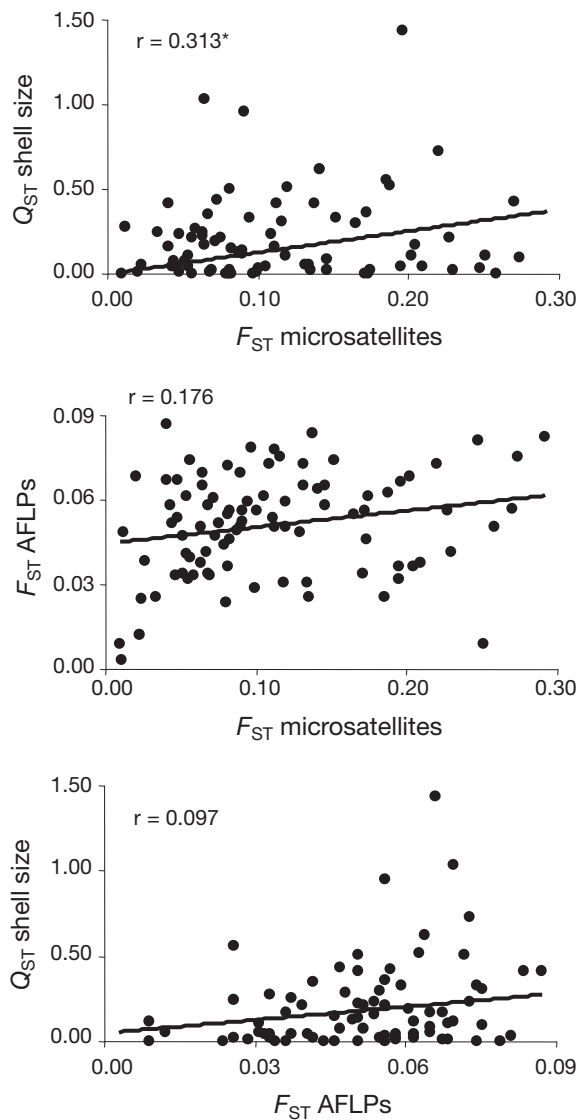| Marker		A(R)	
	R	North	South
Microsatellites	0.052***	0.057***	0.038***
AFLPs	0.022***	0.052***	0.059***
Shell size	0.083***	0.040***	0.002

Fig. 5. *Littorina saxatilis*. Covariation among markers. Pairwise comparison of genetic differentiation among genetic markers. p-values were determined using a Mantel test with 10 000 randomizations. *p < 0.05
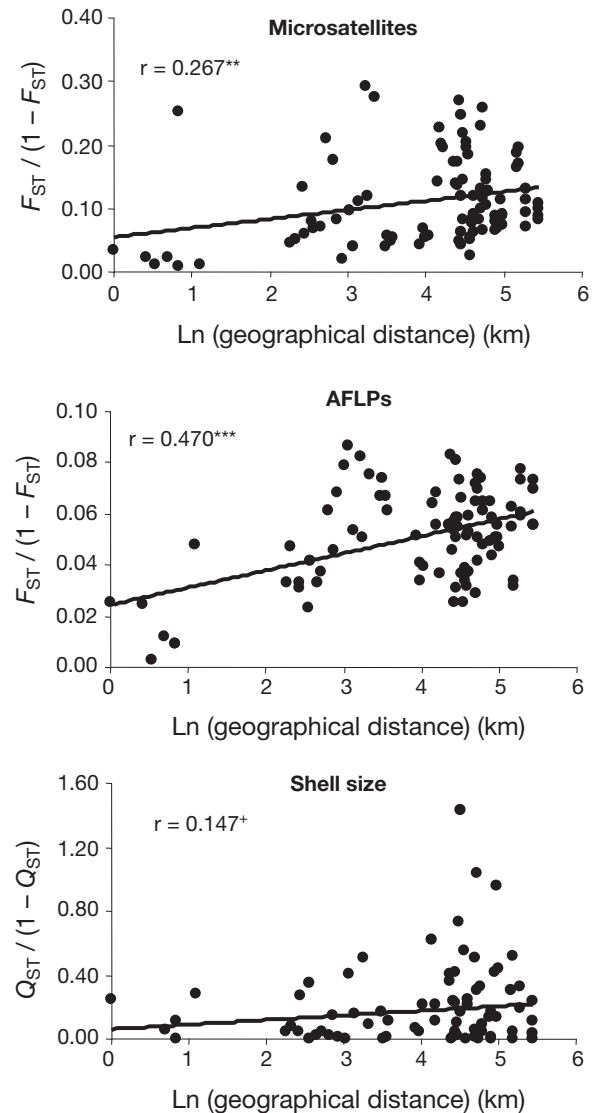

Fig. 6. *Littorina saxatilis*. Isolation by distance. Pairwise comparisons of genetic differentiation in different genetic markers against geographical distance. **p < 0.01, ***p < 0.001, +p < 0.10

DISCUSSION

Geographic adaptation, genetic drift and migration can all have profound effects on the genome-wide patterns of variability of a species. To assess the impact of these forces in the genome of *Littorina saxatilis*, we examined patterns of polymorphism and genetic differentiation using 3 different genetic markers: AFLPs, microsatellites and shell size. The data analysis presented in the present study strongly supports the hypothesis that population structuring in *L. saxatilis* is strongly influenced by an ecological barrier to gene flow in NW Spain. This is supported by the observation that all 3 markers

show a concordant genetic break that coincides in position with an abrupt change in climate, ecological characteristics and oceanographic circulation patterns. The combined influence of these water currents and ecological gradients is probably a major factor responsible for the position and maintenance of the genetic discontinuity. To our knowledge, the current study is the first showing a separation between northern and southern populations at Cape Finisterre, as this boundary has never before been recognized as a genetic barrier.

The heterozygote deficit observed in the present study for microsatellites is a common phenomenon in populations of marine mollusks (Zouros & Foltz 1984) and has

been recurrently observed in *Littorina saxatilis* for allozyme and microsatellite loci (Ward et al. 1986, Panova et al. 2006). Heterozygote deficits are commonly attributed to technical errors. However, MICROCHECKER showed no indication that genotyping errors affected allele scoring (e.g. allele dropouts or stuttering) at any of the microsatellites in any of the samples, although these loci might be influenced by one or more null alleles. High frequency of null alleles may complicate population genetic analyses that rely on Hardy-Weinberg equilibrium because false homozygotes would be common (e.g. de Sousa et al. 2005). Nevertheless, samples that failed to amplify were rare, thus indicating that null homozygotes were not common in our samples. Therefore, adjusting the data set with MICROCHECKER to take into account the putative presence of null alleles did not modify the results obtained. No sample failed to amplify at more than one locus, making it unlikely that poor DNA quality affected our results. Causes for a heterozygote deficit, other than from technical errors, generally include substructuring of the population sample, inbreeding or hitchhiking with a selected locus (e.g. Zouros & Foltz 1984). Interestingly, F_{IS} was found to increase across all loci from unpooled samples (avg. F_{IS} = 0.104, Table 2) to pooled samples (avg. F_{IS} = 0.245, Table 1). This result suggests that a likely cause of the heterozygote deficit could be a micro-spatial Wahlund effect generated by pooling samples with a cryptic population structure.

It is difficult to determine with certainty if the observed genetic break was generated in allopatry or whether these differences were generated in sympatry by natural selection. Indeed, the 2 biogeographic regions represent habitats sufficiently divergent to lead to the observed differentiation through disruptive selection. Alternatively, NW Spain is a biogeographic border where many differentiated populations could presumably meet. Under the last scenario, the most parsimonious explanation for the persistence of the genetic break is that differences between allopatrically diverged populations are being homogenized very slowly after secondary contact in NW Spain.

Several aspects of the data, however, cannot be explained by this biogeographic boundary alone. In particular, the observation of an increased genetic differentiation with geographical distance suggests an isolation-by-distance model of genetic exchange. This result is consistent with very limited migration and indicates that a pattern of isolation-by-distance may be compatible with the presence of a major ecological barrier to gene flow. Thus, whereas limited adult dispersal is likely restricting gene flow among neighboring populations at a local scale, the biogeographic boundary at Cape Finisterre appears to be the major barrier to gene flow at a macro-geographical scale.

Another striking result that apparently deviates from a simple ecological barrier scenario is that AFLP loci displayed a slightly different picture of genetic differentiation among samples from that observed for microsatellites. This distinctive variation includes a generally higher differentiation between areas than among regions and a pattern of pairwise differentiation among samples uncoupled with that observed for microsatellites. Such discrepancies among different kinds of molecular markers are often observed in comparative studies (e.g. Gaudeul et al. 2004). However, genomic sampling error rather than selection is commonly invoked to explain these discrepancies (Bierne et al. 2003). For example, if only a small number of neutral loci are compared between populations, it is likely that one or a few loci display extreme patterns of population differentiation just by neutral genetic drift. In the present study, 3 microsatellite loci are probably not enough to provide an exact view of the whole genome. Thus, in this case AFLPs could be more reliable than microsatellites, and the discrepancy between AFLPs and microsatellites could be due to the better genome coverage provided by the 840 AFLP loci. Another plausible explanation for the lack of concordance between AFLPs and microsatellite markers is that they may suffer different levels of homoplasy, which occurs when alleles identical in state are not identical by descent (Estoup et al. 2002). For both types of markers homoplasy probably increases with genetic divergence between samples, thus biasing downward the estimates of genetic differentiation (Estoup et al. 2002, Vekemans et al. 2002). Therefore, homoplasy is expected to have a greater effect on AFLPs when many loci are scored per primer combination (more than 200 in our case) due to the lack of homology of comigrating fragments (Vekemans et al. 2002). Hence, in the present study, the generally higher differentiation between areas than among regions for AFLP loci suggests higher homoplasy of AFLPs compared to microsatellites.

Genetic or ecological causes (or a combination of both) could also explain the shell size differences among populations on both sides of Cape Finisterre. It might be argued that mating asynchrony could be responsible for the different shell sizes of embryos collected among the 2 regions. According to this hypothesis, variation in shell size across redions would be the result of differences in average embryo age. This explanation, however, is unlikely since *Littorina saxatilis* populations from NW Spain mate and produce embryos during the entire year (Reid 1996). Consistent with this, all females examined in the present study had embryos in their embryo pouches, indicating a low frequency of non-mated females in populations from

each region. We note that females contain embryos at different developing stages, but we only extracted developed embryos (shell height >0.5 mm) from females randomly chosen from each sampling site. This supports the view that genetically based differences are at least partially involved in the observed pattern of quantitative variation.

Genetic differentiation between subpopulations coincident in position with a biogeographic boundary has been observed in several other marine species (reviewed in Avise 2004). In many cases, local adaptation and distinct hydrographic features were proposed as causal agents in the breakdown of gene flow (e.g. Quesada et al. 1995). For example, the long-term climate and topographical changes in coastal California have been posited as a major factor influencing the evolution of marine organisms (Burton & Lee 1994). The discovery in this area of a close concordance among these environmental changes with the phylogeographic and biogeographic patterns of marine invertebrates and fishes suggested that similar processes influenced the evolution of coastal marine taxa (Dawson 2001). Our results suggest that the observed patterns of differentiation may be maintained by environmentally induced restrictions in gene flow that allow genetic drift, and perhaps natural selection, to cause the observed genetic differentiation. Further studies on other widely distributed species will shed light on the origin of the differences detected in *Littorina saxatilis*. If genetic surveys of additional taxa reveal other examples of northern–southern genetic discontinuities in NW Spain, this pattern would point to a historical factor affecting many species.

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