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No evidence for genetic differentiation between Antarctic limpet *Nacella concinna* morphotypes

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Abstract The extent to which genetic divergence can occur in the absence of physical barriers to gene flow is currently one of the most controversial topics in evolutionary biology, with implications for our understanding of speciation, phenotypic plasticity and adaptive potential. This is illustrated by a recent study reporting a surprising pattern of genetic differentiation between intertidal and subtidal morphotypes of the broadcast-spawning Antarctic limpet *Nacella concinna*. To explore this further, we collected almost 400 Antarctic limpets from four depths (intertidal, 6, 15 and 25 m) at Adelaide island, Antarctica, and conducted a combined morphometric and genetic analysis using 168 polymorphic amplified fragment length polymorphism (AFLP) loci. Morphological analysis revealed not only pronounced differences between the two morphotypes, but also a continuous cline in shell shape from the intertidal zone down to 25 m depth, suggesting that the distinction between the morphotypes may be artificial. Moreover, genetic analysis using both F_{st} and a Bayesian analogue found no evidence for differentiation either between the two morphotypes or by depth, and a Bayesian cluster analysis did not detect any cryptic genetic structure. Our findings lend support to the notion that limpets can be phenotypically highly plastic, although further studies are required to determine unequivocally whether there is any genetic basis to the observed variation in shell morphology.

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Introduction

Marine rocky shores are highly heterogeneous, physically stressful environments characterised by strong environmental gradients in a variety of abiotic variables including temperature, ultraviolet irradiation, salinity and wave action (Menge and Branch 2001). These gradients not only shape community structure but are also capable of generating strong patterns of divergent selection within species, with the potential to initiate genetic divergence even in the face of substantial gene flow. An interesting example (reviewed by Johannesson 2003; Butlin et al. 2008) is provided by the periwinkle *Littorina saxatilis*, a direct-developing gastropod that inhabits rocky shores throughout Europe. This species possesses two distinct morphotypes: a large, thick-shelled form with a narrow aperture that is adapted to crab predation at sheltered sites and a smaller, thin-shelled morph with a larger foot area that is found in more wave-exposed areas where crabs are largely absent. Here, strong disruptive selection appears to have generated partial barriers to gene flow (e.g. Wilding et al. 2001; Rolán-Alvarez et al. 2004; Panova et al. 2006; Galindo et al. 2009) that are reinforced by assortative mating (Pickles and Grahame 1999; Rolán-Alvarez et al. 1999; Hollander et al. 2005).

In contrast, although gamete recognition proteins might in principle allow assortative mating in broadcast-spawning species (Palumbi 1999; Swanson et al. 2001), it is unknown how widely this might occur. Moreover, long-lived planktonic larvae facilitate long-distance transportation by ocean currents but also greatly reduce the potential for fine-scale

genetic structure. Consequently, it is widely believed that broadcast-spawners recruit from a common gene pool, thereby constraining them to adapt to local conditions through phenotypic plasticity (Johannesson 2003). This is consistent with two studies of the planktonic-developing periwinkle *Littorina striata* (now a synonym of *Tectarius striatus*) that were unable to distinguish genetically between two different shell morphotypes; a nodulose form inhabiting wave-sheltered sites and a smooth form dominating more exposed shores (de Wolf et al. 1998a; de Wolf et al. 1998b). However, a recent genetic study of the Antarctic limpet *Nacella concinna* (Strebel 1908) by de Aranzamendi et al. (2008) suggests that this generalisation might not hold for all broadcast-spawning species.

Nacella concinna is one of the most abundant invertebrates found in the Antarctic marine environment, occurring from the intertidal zone (Walker 1972) down to depths of over 100 m (Powell 1951). This species is a broadcast-spawner that reproduces between mid November and mid December (Picken 1980, Stanwell-Smith and Clarke 1998). Juvenile recruitment occurs from late January through to March, the eggs require around 8 days from fertilisation to hatching at 0°C and the free-swimming planktonic veliger larval stage lasts for 1–2 months (Bowden et al. 2006). Two distinct *N. concinna* morphotypes have long been recognised; an intertidal '*polaris*' form and a subtidal '*concinna*' type found below 4 m depth (Strebel 1908; Powell 1951). The intertidal morphotype has a taller and heavier shell, whereas the subtidal morphotype has a lighter, flatter shell that is usually microscopically 'scalloped' due to grazing by other limpets on encrusting endolithic algae (Strebel 1908; Powell 1951; Nolan 1991; De Aranzamendi et al. 2008; Weihe and Abele 2008). The two morphotypes also differ in important physiological traits such as tolerance to experimental deep freezing (Waller et al. 2006) and metabolic response to air exposure (Weihe and Abele 2008). These differences represent a remarkable suite of adaptations to a strong environmental gradient characterised by variation in periodic tidal emersion, ambient temperature, humidity, oxygen availability, ultraviolet irradiation and physical disturbance from ice (Menge and Branch 2001; Peck et al. 2006).

Numerous authors have speculated that the two morphotypes could be genetically distinct populations (e.g. Nolan 1991; Beaumont and Wei 1991; de Aranzamendi et al. 2008; Weihe and Abele 2008). However, this seems unlikely given this species' broadcast-spawning life history and the fact that considerable levels of phenotypic plasticity have been reported in several temperate limpet species (e.g. Moore 1934). Moreover, the intertidal morphotype is also migratory, moving seasonally between the intertidal and subtidal zones (Walker 1972). Nevertheless, two independent molecular studies have sought to determine whether the two morphotypes are genetically differentiated. The first by Beaumont and Wei (1991) used five polymorphic allozyme loci to compare intertidal and subtidal populations from the South Orkney Islands and Stromness, South Georgia. No differences were found between the two morphotypes, but this could be attributable to low levels of genetic polymorphism, with the loci used possessing on average only three alleles. In contrast, a more recent study by de Aranzamendi et al. (2008) using inter-simple sequence repeat (ISSR) markers reported statistically significant genetic differences between the two morphotypes when sampled from three populations within Potter Cove on the South Shetland Islands. To account for their results, they proposed an intriguing mechanism in which, although the two morphotypes have overlapping spatial distributions for much of the year, vertical migration of the intertidal morphotype from the subtidal into the intertidal zone prior to reproduction prevents cross-fertilisation from occurring. These authors further speculated that the magnitude of genetic divergence between the two morphotypes should vary with the heritability of migratory behaviour, with high heritability favouring the fixation of genetic differences and low heritability allowing gene flow and thus maintaining species cohesion.

If verifiable, de Aranzamendi et al.'s (2008) finding would make an important contribution to our understanding of non-allopatric genetic divergence. However, with very little being known about either larval behaviour or the extent to which water layers from different depths mix, it is unclear whether vertical separation at spawning could present a sufficiently strong barrier to gene flow to allow the two morphotypes to diverge. Moreover, de Aranzamendi et al.'s (2008) sample size was small, comprising a total of 108 individuals distributed over three populations and three depths, and only 35 polymorphic binary loci were analysed. The reported genetic differences between the two morphotypes were also undetectable in all but one of the populations using F_{st} and could only be found using a Bayesian approach.

Amplified fragment length polymorphisms (AFLPs) are well suited to the study of population structure, being predominantly nuclear in origin and readily amplifiable across a range of both prokaryotic and eukaryotic taxa. Importantly, they combine high reproducibility with the potential to screen large numbers of loci (Vos et al. 1995; Mueller and Wolfenbarger 1999; Bensch and Akesson 2005) and are also capable of resolving even extremely small genetic differences (Maughan et al. 1996). Consequently, they are rapidly becoming one of the most popular tools for genetic analysis in the fields of evolutionary and conservation genetics (Caballero et al. 2008).

Herein, we report the results of a combined morphological and AFLP analysis of almost 400 *N. concinna* individuals

sampled at Adelaide Island, Antarctica. Our primary aim was to determine whether the two morphotypes represent genetically divergent populations, as suggested by de Aranzamendi et al. (2008). However, previous authors proposed the existence of the two morphotypes on the basis of only comparing samples from intertidal and subtidal localities (Strebel 1908; Powell 1951), whereas physiological differences have also been observed between subtidal limpets from 6 m and 25 m depth (S. Morley, personal communication). Therefore, to allow the evaluation of ecological factors that vary with depth both across the intertidal zone and subtidally, we sampled individuals from four different depths along a linear transect. We then tested for genetic differentiation both between the intertidal and subtidal morphotypes and also by depth.

Materials and methods

Study site and specimens

The aim of this study was to analyse not only the two distinct morphotypes, but also to study a more extensive depth profile. Consequently, we collected 96 *N. concinna* individuals from each of four different populations (total $n = 384$ specimens) along a linear transect at South Cove, adjacent to Rothera research station on Adelaide island on the Antarctic Peninsula (Fig. 1). The intertidal morphotype was sampled from the intertidal zone, while the subtidal morphotype was represented by three different populations collected at 6, 15 and 25 m depths. The specimens were collected during the austral summer of 2008 either by hand from the inter-tidal region or by SCUBA divers. The animals were immediately shucked, foot tissues were excised from each limpet and stored in 95% ethanol for subsequent genetic analysis and the shells were allowed to dry overnight and stored in individually labelled plastic bags.

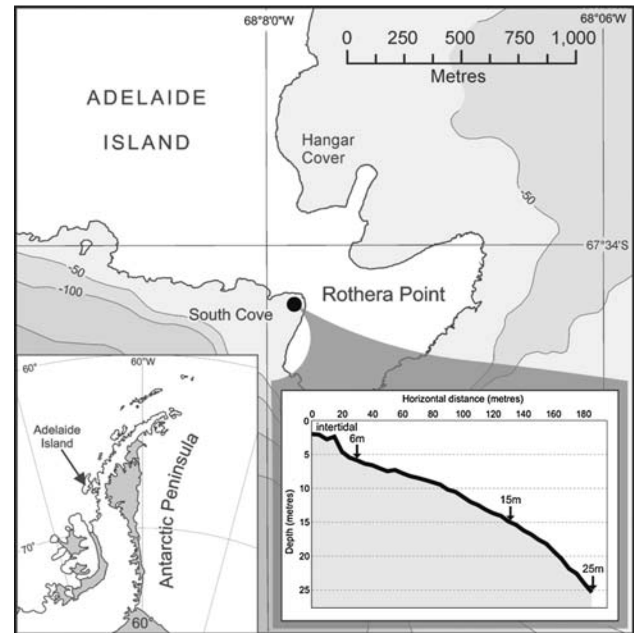

Fig. 1 *Map* showing the location of the *Nacella concinna* sampling transect at Rothera Point, Adelaide Island. The *bottom-right inset* shows the depth profile along which the samples were collected

Collection and analysis of morphological data

Shell length (L), breadth (B) and height (H) were measured to $\pm$ 0.01 mm using vernier callipers and shell dry mass (M) was recorded to the nearest 0.1 mg. Shell thickness (T) was then estimated as M divided by shell surface area, calculated by applying the standard formula for a cone using the above measurements. To simultaneously visualise both allometric and depth-related patterns of morphological variation, L was plotted against M, H, T and B on a log–log scale. Next, to determine whether any of these parameters differed significantly between the two morphotypes, we used the program R (R development team 2005) to construct general linear models (GLMs), fitting L as a continuous variable, morphotype as a factor with two levels, and the L: morphotype interaction. H, M and T were log transformed prior to analysis, which improved normality. Using standard deletion-testing procedures (Crawley 2002), each term was then sequentially dropped from a model unless doing so significantly reduced the amount of deviance explained (deviance is analogous to sums of squares in standard regression analysis). The change in deviance between full and reduced models should be distributed as χ^2 with degrees of freedom equal to the difference in degrees of freedom between the models with and without the term in question. Distributions of standardised residuals about regressions were inspected to verify that they were approximately normally distributed. These analyses were also repeated after restricting the dataset to the three subtidal populations, this time fitting L as a continuous variable, depth as a factor with three levels, and the L: depth interaction.

To compare the two-dimensional shell outlines of the individuals, we next used Fourier shape analysis as developed and explained by Crampton and Haines (1996). This approach decomposes an outline contour into a number of basic waves termed harmonics, each of which is in turn explained by two respective Fourier coefficients. The more harmonics, and therefore Fourier coefficients calculated, the more accurate the description of the outline will be. Each shell outline is so described by a set of Fourier coefficients that can be statistically treated as any usual variable. Since Fourier coefficients contain no size information, a standardisation of the outlines to the same size is not

necessary. For this analysis, digital photographs of all specimens were taken. After enhancing the contrast between the shell and its background, shell outlines were digitised using the program ImageJ (Rasband 2008). The program Htree was used to rotate all of the outlines to allow maximum overlap as strongly recommended by Haines and Crampton (2000) for organisms such as limpets that are difficult to orient in a homologous way. After applying a smoothing normalisation of 20 to eliminate any high-frequency pixel noise resulting from the digitisation process, the digitised outlines were then subject to fast Fourier transformation using program Hangle (Crampton and Haines 1996). The shell outlines were captured with sufficiently high precision using the first eight harmonics. However, since the first of these does not contain any shape information and is thus discarded from the analysis, a set of 14 Fourier coefficients per individual was obtained. To visualise the shape information contained within the Fourier coefficients, these were subject to principal component analyses (PCA) based on the variance–covariance matrix using the program Past (Hammer et al. 2001). The resulting PC axes were cast in units of standard deviation and centred about the overall mean (see Crampton and Maxwell 2000). The number of principal components to be retained was determined by use of the Kaiser criterion (Kaiser 1960). To facilitate interpretation, a scatter plot of the population centroids of the first two principal components (PCs) was constructed and synthetic outlines of 'extreme shell forms' were generated using Htree following Crampton and Haines (1996).

Collection of genetic data

Total genomic DNA was first extracted from a small piece of foot tissue using the Qiagen DNeasy tissue extraction kit following the manufacturer's recommended protocols. The AFLP protocol was similar to that used by Vos et al. (1995) and Ajmone-Marsan et al. (1997) and is described in detail by Dasmahapatra et al. (2009). Briefly, 100–400 ng of genomic DNA was first digested using *Taq*I (5U in a 10 µl volume at 65°C for 2 h) and then with *Eco*RI (5U in a 20 µl volume at 37°C for 2 h). *Taq*I and *Eco*RI and adapters (Ajmone-Marsan et al. 1997) were then ligated onto the digested DNA using T4 DNA ligase (1U in a 50 µl volume at 37°C for 3 h), and the resulting products diluted by a factor of 10 in 10 mM Tris–HCL and EDTA (0.1 mM, Ph 8.0). For the pre-amplification, 5 µl of ligation mix was added to a 50-µl PCR reaction containing Tris–HCl (10 mM, pH 8.3), $MgCl_2$ (1.5 mM), KCl (50 mM), dNTPs (0.2 mM), *Taq* polymerase (1U) and 50 ng each of the *Taq*I-C and *Eco*RI-A pre-amplification primers (the primer sequences were 5′-GATGAGTCCTGACCGAC-3′ and 5′-GACTGCGTACCAATTCA-3′, respectively). Following 30 pre-amplification cycles (30 s at 94°C, 60 s at 50°C and 60 s at 72°C), the products were diluted 10 times with 10 mM Tris–HCl and EDTA (0.1 mM, Ph 8.0). For the selective amplification, 2.5 µl of the diluted pre-amplification product was added to a 12.5-µl reaction containing Tris–HCl (10 mM, pH 8.3), $MgCl_2$ (1.5 mM), KCl (50 mM), dATPs, dTTP and dGTP (0.2 mM each), dCTP (0.04 mM), α^{33}P-dCTP, *Taq* polymerase (0.2U), *Taq*I selective primer (30 ng) and *Eco*RI selective primer (5 ng). Samples were subjected to 13 selective amplification cycles (30 s at 94°C, 60 s at 65°C, reducing by 0.7°C each cycle and 60 s at 72°C), followed by further 23 cycles (30 s at 94°C, 60 s at 56°C and 60 s at 72°C). Seven different selective primer combinations were used (Table 1). PCR products were resolved by electrophoresis on standard 6% polyacrylamide sequencing gels and detected by autoradiography. Exposed X-ray films were assessed, and if required, a second exposure was made for an adjusted time period. Gels were scored by eye and genotypes were entered manually into a Microsoft excel spreadsheet.

Quantification of the genotyping error rate

Although AFLPs tend to be highly reproducible due to the use of highly specific restriction endonucleases coupled with stringent PCR conditions, (Vos et al. 1995; Bonin

Table 1 Primer combinations used for the AFLP selective amplification and numbers of AFLP polymorphisms generated for 384 *N. concinna* individuals

*Taq*I primer (5′–3′)	*Eco*RI primer (5′–3′)	Total number loci	Number of polymorphic loci	Polymorphism (%)
GATGAGTCCTGACCGACTG	GACTGCGTACCAATTCAGC	39	32	82.1
GATGAGTCCTGACCGACGA	GACTGCGTACCAATTCAGC	30	21	70.0
GATGAGTCCTGACCGACAG	GACTGCGTACCAATTCAGC	7	4	57.1
GATGAGTCCTGACCGACAC	GACTGCGTACCAATTCAGC	32	25	78.1
GATGAGTCCTGACCGACAC	GACTGCGTACCAATTCATG	39	33	84.6
GATGAGTCCTGACCGACCA	GACTGCGTACCAATTCAAC	33	26	78.8
GATGAGTCCTGACCGACCA	GACTGCGTACCAATTCACA	30	27	90.0
Total		210	168	80.0

et al. 2004; Meudt and Clarke 2007) genotyping errors can nevertheless accrue, with potential sources including DNA contamination (Dyer and Leonard 2000), restriction artefacts (Polisky et al. 1975) and human error (Bonin et al. 2004). Consequently, we estimated the genotyping error rate for our dataset by independently re-extracting, re-genotyping and blind-scoring over 10% of the samples ($n = 40$ individuals) following Hoffman and Amos (2005). To ensure broad coverage of the dataset, ten individuals were selected at random from each of the four populations. The error rate per reaction was then quantified as the number of mismatching genotypes divided by the number of polymorphic bands compared (Bonin et al. 2004).

Genetic data analysis

The AFLP dataset analysed consisted of 64,512 binary character reads representing the presence and absence genotypes of 384 individuals at 168 polymorphic AFLP loci. To facilitate comparison with the results of de Aranzamendi et al. (2008), we followed their approach of employing both traditional and Bayesian estimators of genetic differentiation. First, we used the program Aflp-Surv v1.0 (Vekemans 2002) to calculate pairwise F_{st} values (Weir and Cockerham 1984) following the approach of Lynch and Milligan (1994) between the two morphotypes and also among the four populations sampled from different depths. This program was also used to conduct a permutation test for overall genetic differentiation using 10,000 permutations of the dataset.

Traditional approaches for estimating F_{st} using dominant markers can sometimes prove problematic owing to the assumption that populations conform to Hardy–Weinberg equilibrium. Moreover, biased estimates of F_{is} (population inbreeding coefficient) can be obtained using dominant markers, particularly when sample sizes are small (Lynch and Milligan 1994; Zhivotovsky 1999). Although these biases appear to be largely eliminated when reasonable numbers of polymorphic loci are used (Krauss 2000), we nevertheless felt it prudent to apply the approach of Holsinger et al. (2002) as implemented in the program Hickory v1.1 to estimate Bayesian analogues of F_{st} and F_{is}, designated θ^{II} and f, respectively. This software allows the estimation of four different models (Holsinger and Wallace 2004). The first is a full model in which θ^{II} and f are estimated simultaneously. Two alternative models assume that $f = 0$ (no inbreeding within populations) and $\theta^{II} = 0$ (no differentiation among populations), respectively. Finally, because estimates of f can be unreliable for dominant datasets, the program allows the estimation of a final model in which f is free to vary. All four models were run for the entire dataset plus each of the pairwise population comparisons, using a burn-in of 50,000 iterations followed by a further 250,000 iterations, with values being retained at every 50th iteration as recommended by Holsinger et al. (2002). Posterior distributions and trace plots were carefully scrutinised to ensure convergence of the Markov chain Monte Carlo sampler. Models were then compared using the deviance information criterion (DIC) which is analogous to Akaike's information criterion (AIC) and combines a measure of model fit (Dbar) with one of model complexity (pD, the effective number of parameters). Models with smaller DIC values are preferred, but a difference of more than six units is required to indicate strong support for one model over another (Holsinger et al. 2002).

We finally conducted a Bayesian cluster analysis of the AFLP dataset using the program Structure, which is described in detail by Pritchard et al. (2000) and Falush et al. (2003). We specifically employed a recent version of the program (version 2.2.3) designed to handle dominant data (Falush et al. 2007). Structure uses multilocus genotype data to cluster individuals into K populations without prior knowledge of population membership. The estimated log probability of the data, denoted ln $P(D)$, is calculated for each value of K, allowing estimation of the most likely number of populations. The membership of each individual in a population is then estimated as q, which varies between 0 and 1 with the latter indicating full population membership. We ran five independent runs for $K = 1$–4 using 1×10^6 MCMC iterations after a burn-in of 1×10^5, specifying the correlated allele frequencies model and assuming admixture. Structure has been extensively validated by the authors (Pritchard et al. 2000; Falush et al. 2003; Falush et al. 2007) and has also been shown elsewhere to be highly effective at identifying the correct number of populations, even when F_{st} is very low (Latch et al. 2006).

Results

Morphological differences between the two morphotypes

In support of previous studies, clear morphological differences were found between the shells of the intertidal and subtidal Antarctic limpet morphotypes (Fig. 2; Table 2). One of the most striking differences was detected in shell dry mass (M), with the shells of intertidal limpets being on average over twice as heavy as those of subtidal limpets (Fig. 2a). To explicitly analyse morphotype-related differences in M while controlling for shell length (L), we constructed a GLM of M, fitting L as a continuous predictor variable together with morphotype as a binary factor and the L:morphotype interaction. Both L and depth were retained in the final model (Table 2a), suggesting that intertidal shells are heavier even after controlling for body size. In addition, the L: morphotype interaction was also retained

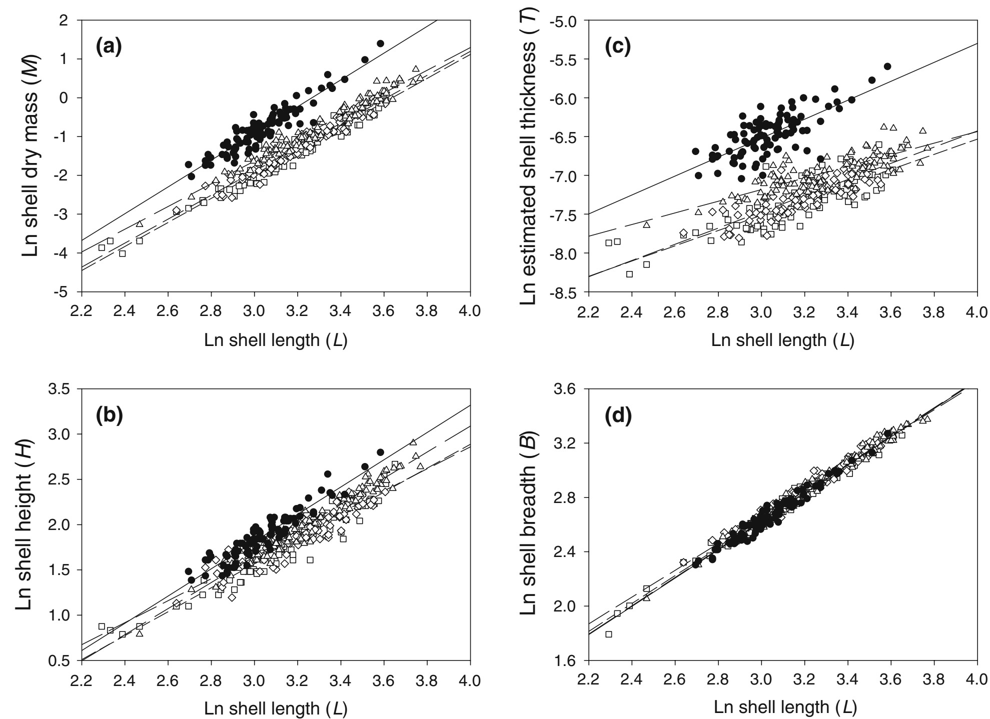

Fig. 2 Patterns of morphological variation among *Nacella concinna* individuals sampled from four different depths. Shell measurements were recorded in millimetres. *Filled circles* represent the intertidal morphotype and empty symbols the subtidal morphotype (triangles = 6 m depth, diamonds = 15 m depth and squares = 25 m depth). Shell length (*L*) is plotted against (**a**) shell dry mass (*M*); (**b**) shell height (*H*); (**c**) estimated shell thickness (*T*); (**d**) shell breadth (*B*)

Table 2 Results of general linear models (GLMs) of shell mass (*M*), height (*H*), thickness (*T*) and breadth (*B*) comparing the two *N. concinna* morphotypes

Term	Estimate	χ^2	*df*	*P*
(a) Shell mass (*M*) *N* = 384 individuals, total deviance = 238.2, explained deviance = 91.2%				
L	0.15	1,870.0	1	<0.0001
Morphotype	–	563.0	1	<0.0001
L: morphotype	–	15.5	1	<0.0001
(b) Shell height (*H*) *N* = 384 individuals, total deviance = 46.0, explained deviance = 88.9%				
L	0.07	1,514.0	1	<0.0001
Morphotype	–	117.3	1	<0.0001
L: morphotype	–	12.0	1	0.00059
(c) Shell thickness (*T*) *N* = 384 individuals, total deviance = 73.2, explained deviance = 80.4%				
L	0.054	2,88.5	1	<0.0001
Morphotype	–	706.8	1	<0.0001
L: morphotype	–	6.3	1	0.012
(d) Shell breadth (*B*) *N* = 384 individuals, total deviance = 7,456.2, explained deviance = 98.0%				
L		8,250.3	1	<0.0001
Morphotype	–	4.6	1	0.01
L: morphotype	–	0.3	1	0.59

Explanatory variables fitted in the full models were shell length (*L*), morphotype as a binary factor and the *L*: morphotype interaction. The χ^2 values for each term represent the change in deviance after removing that term and all interactions involving that term from the model. *df* degrees of freedom

in the model, implying that the slope of the relationship between *L* and *M* differs significantly between the two morphotypes. Quantitatively very similar results were also obtained for shell height (*H*, Fig. 2b; Table 2b) and shell thickness (*T*, Fig. 2c; Table 2c) indicating that intertidal animals also possess relatively tall and thick shells.

More subtle shell shape differences were detected between the two morphotypes using a GLM of shell breadth (*B*) in which we fitted *L*, morphotype and the *L*: morphotype interaction. Although not readily evident from Fig. 2d, morphotype was retained in the final model together with *L* (Table 2d), indicating a subtle effect of depth on the ratio of *L*:*B*. To explore this further, we conducted a Fourier analysis of digitised shell outlines and subjected the resulting coefficients to PCA. PC1, PC2 and PC3 were retained in the analysis on the basis of the Kaiser criterion, accounting for 17.8, 13.0 and 10.6% of the variation in shell outline shape respectively. Intertidal limpets possessed significantly higher PC1 values on average than subtidal individuals (Fig. 3, ANOVA, $F_{1,382} = 85.18$, $P < 0.0001$), while PC2 and PC3 showed no differences among the populations. The synthetic outlines shown in Fig. 3 reveal a tendency for subtidal shells tend to be 'pear-shaped', whereas intertidal shells are more quadrangular in outline.

Since physiological differences have previously been observed between limpets sampled from 6 m and 25 m depth (S. Morley, personal communication), we next analysed morphological data from three subtidal populations (sampled from 6 m, 15 m and 25 m depths). To test whether *M* varied significantly among these populations, we constructed a GLM, fitting *L* as a continuous predictor variable together with depth as a factor with three levels and the *L*: depth interaction. All three of these predictors were retained (Table 3a), indicating that *M* not only differs between the two morphotypes but also varies with depth among the subtidal populations. Similar results were also obtained for *H* (Table 3b), *T* (Table 3c) and *B* (Table 3d).

Population genetic structure

To test for genetic differentiation, we genotyped a total of 384 individuals at seven different selective primer combinations, yielding 210 AFLP loci that could be scored unambiguously across all of the samples, of which 168 (80.0%) were polymorphic (Table 1). High-quality banding patterns were obtained and consequently the genotyping error rate, assessed by re-extracting, genotyping and blind-scoring over 10% of all individuals, was low at 0.008 (57 differences observed out of 6,720 comparisons). Of the discrepancies observed between the two sets of genotypes, 22 (36.6%) were attributed to scoring or data entry errors and the remaining 35 (61.4%) were due to the stochastic

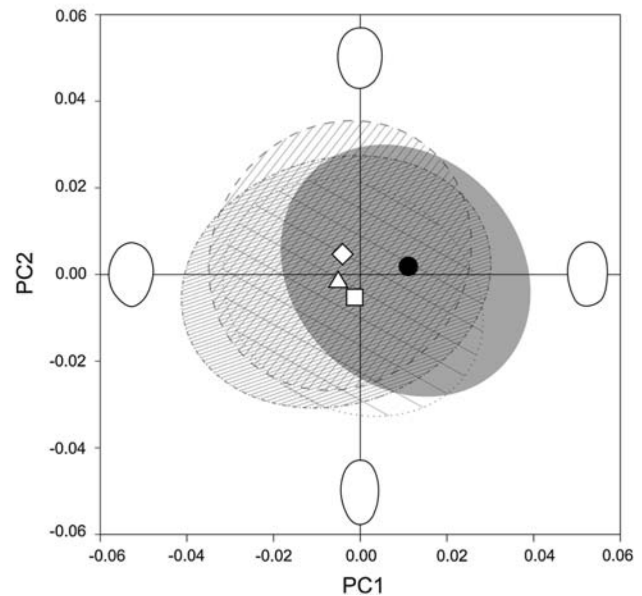

Fig. 3 Mean PC1 and PC2 values (centroids) and 95% confidence ellipses of four *Nacella concinna* populations derived from Fourier shape analysis depicting shell outline differences among individuals. Intertidal morphotype = *filled circle* with *grey-shaded* ellipse; 6-m depth = *empty triangle* with *fine-hatched ellipse*; 15-m depth = *empty diamond* with *medium-hatched ellipse*; 25-m depth = *empty square* with *coarse-hatched ellipse*. PC1 and PC2 explain 17.8 and 13.0% of the variation in shell *outline shape*, respectively. *Synthetic outlines* are shown that represent the hypothetical shell outlines of animals with extreme PC1 and PC2 values (−0.05 and +0.05), with the *anterior margin* facing the *bottom* of the page and the *right margin* facing the *left*

appearance or disappearance of bands as similarly documented by Bonin et al. (2004).

A significance test for genetic differentiation between the intertidal and subtidal morphotypes using 10,000 permutations of the dataset found no evidence for divergence between the two forms ($F_{st} = -0.0002$, 95% CI = −0.0016–0.0016, $P = 0.49$). Similarly, when the dataset was partitioned into the four populations sampled from different depths, no genetic differences were apparent (overall $F_{st} = -0.0003$, 95% CI = −0.0016–0.0010, $P = 0.56$). Similarly, pairwise F_{st} values among the populations were all low, with three comparisons yielding values of zero and the others yielding values less than or equal to 0.0004, none of which were statistically significant.

Because traditional F_{st} estimation methods rely upon assumptions about populations being in Hardy–Weinberg equilibrium, we next sought to either confirm or refute the above findings by conducting a Bayesian analysis of population genetic structure using the program Hickory (Holsinger et al. 2002). The *f* and θ^{II} values obtained from four different models of population structure are shown in Table 4. For the comparison of the intertidal and subtidal morphotypes, the $f = 0$ model yielded the lowest Dbar

Table 3 Results of general linear models (GLMs) of shell mass (*M*), height (*H*), thickness (*T*) and breadth (*B*) comparing the three subtidal *N. concinna* populations

Term	Estimate	χ^2	*df*	*P*
(a) Shell mass (*M*) *N* = 288 individuals, total deviance = 193.50, explained deviance = 94.0%				
L	0.11	1,401.5	1	<0.0001
Depth	–	38.6	2	<0.0001
L:depth	–	23.1	2	<0.0001
(b) Shell height (*H*) *N* = 288 individuals, total deviance = 38.93, explained deviance = 91.19%				
L	0.05	879.9	1	<0.0001
Depth	–	13.9	2	<0.0001
L:depth	–	4.9	2	0.0079
(c) Shell thickness (*T*) *N* = 288 individuals, total deviance = 28.98, explained deviance = 76.95%				
L	0.03	211.9	1	<0.0001
Depth	–	45.7	2	<0.0001
L:depth	–	14.5	2	<0.0001
(d) Shell breadth (*B*) *N* = 288 individuals, total deviance = 5846.24, explained deviance = 98.01%				
L	0.73	4,459.4	1	<0.0001
Depth	–	2.8	2	0.026
L:depth	–	1.4	2	0.25

Explanatory variables fitted in the full models were shell length (*L*), depth as a factor with three levels and the *L*: depth interaction. The χ^2 values for each term represent the change in deviance after removing that term and all interactions involving that term from the model. *df* represents degrees of freedom

value, indicating that this model had the best fit to the data-set and hence that inbreeding is likely to be absent within populations, which is consistent with this species being a broadcast-spawner. In contrast, using the model selection criterion DIC, which takes into account both model fit and the effective number of parameters being estimated, the lowest value was instead obtained for the full model. However, because the differences in DIC between the full, $f = 0$ and $\theta^{II} = 0$ models are small (<3 units) and arise partly due to differences in model dimensionality, there is little reason to prefer any one of the models over the others (Holsinger et al. 2002). Reassuringly, both the full and $f = 0$ models yielded low θ^{II} estimates at 0.00115 (95% CI = 0.000163–0.00295) and 0.000718 (95% CI = 5.15×10^{-5}–0.00186), respectively. Virtually identical results were obtained when all four populations were analysed together, as well as for each of the pairwise population comparisons (Table 4).

Bayesian cluster analysis

Arguably, some of the most powerful tests of population structure need not rely on information about the populations from which individuals were sampled. Consequently, we finally implemented a Bayesian cluster analysis using the program Structure (Pritchard et al. 2000; Falush et al. 2007) to test whether genetic structure could be detected in the absence of a priori population membership data. The resulting posterior probabilities of *K* were highly concordant between replicate runs, with the lowest average value indicating the most likely *K*. In support of the previous analyses, the most likely number of distinct groups present in our sample set was robustly determined as one (ln *P*(*D*) was $-32{,}983 \pm 0.47$ SE for $K = 1$, $-33{,}640 \pm 63.80$ SE for $K = 2$, $-36{,}491 \pm 333.29$ SE for $K = 3$ and $-38{,}666 \pm 640.79$ SE for $K = 4$), providing further evidence for an absence of genetic differentiation either between the two morphotypes or by depth.

Discussion

There is mounting evidence that local adaptation in direct-developing marine gastropods is capable of driving genetic divergence even within continuous populations that are not subdivided by physical barriers. However, despite theoretical expectations to the contrary, it is currently unclear whether non-allopatric genetic divergence occurs in indirect-developing gastropods, with a recent study of the Antarctic limpet *N. concinna* reporting a surprising pattern of genetic differentiation between two morphologically and physiologically distinct morphotypes (de Aranzamendi et al. 2008) despite this species adopting a broadcast-spawning life history. Our study reveals a contrasting picture in which strong depth-related patterns of morphological variation are not accompanied by genetic divergence as measured using 168 polymorphic AFLP loci.

Table 4 Bayesian analysis of *Nacella concinna* population structure using the program Hickory (Holsinger et al. 2002)

Populations compared	Model	f	θ^{II}	95% credibility interval for θ^{II}	Dbar	pD	DIC
Intertidal versus subtidal morphotype	Full	0.979	0.00115	0.000163–0.00295	1,739.73	175.15	1,914.87
	$f = 0$	–	0.000718	5.15×10^{-5}–0.00186	1,738.69	178.54	1,917.22
	$\theta^{II} = 0$	0.979	–	–	1,761.55	154.87	1,916.42
	Free f	0.503	0.0802	0.0294–0.155	2,219.18	722.77	2,941.94
All four populations	Full	0.979	0.000778	4.02×10^{-5}–0.00207	3,106.46	184.98	3,291.44
	$f = 0$	–	0.000553	5.24×10^{-5}–0.00138	3,100.34	192.21	3,292.54
	$\theta^{II} = 0$	0.979	–	–	3,139.54	155.00	3,294.54
	Free f	0.497	0.0413	0.0130–0.0997	3,398.55	833.73	4,232.28
Intertidal versus 6 m	Full	0.974	0.00169	0.000104–0.00459	1,533.74	163.98	1,697.72
	$f = 0$	–	0.00120	9.01×10^{-5}–0.00299	1,528.55	169.97	1,698.52
	$\theta^{II} = 0$	0.975	–	–	1,554.82	145.14	1,699.96
	Free f	0.503	0.0702	0.024276–0.140	1,723.92	447.82	2,171.74
Intertidal versus 15 m	Full	0.978	0.000878	3.68×10^{-5}– 0.00284	1,533.62	156.77	1,690.40
	$f = 0$	–	0.000582	1.79×10^{-5}–0.00183	1,532.2	160.84	1,693.04
	$\theta^{II} = 0$	0.977	–	–	1,543.92	147.38	1,691.29
	Free f	0.499	0.0712	0.0262–0.136	1,735.53	452.80	2,188.33
Intertidal versus 25 m	Full	0.975	0.00213	0.000160–0.00526	1,535.37	167.81	1,703.18
	$f = 0$	–	0.00140	0.000135–0.00329	1,532.84	172.28	1,705.12
	$\theta^{II} = 0$	0.976	–	–	1,563.26	145.56	1,708.82
	Free f	0.500	0.0722	0.0267–0.142	1,721.88	447.35	2,169.24
6 m versus 15 m	Full	0.979	0.00198	0.000130–0.00492	1,553.21	169.00	1,722.2
	$f = 0$	–	0.00127	0.000111–0.003163	1,550.99	173.30	1,724.3
	$\theta^{II} = 0$	0.979	–	–	1,578.55	147.59	1,726.13
	Free f	0.497	0.0739	0.0279–0.143	1,743.29	457.39	2,200.69
6 m versus 25 m	Full	0.977	0.00130	6.95×10^{-5}–0.00385	1,533.86	160.75	1,694.61
	$f = 0$	–	0.000817	5.17×10^{-5}–0.00230	1,532.38	164.32	1,696.7
	$\theta^{II} = 0$	0.977	–	–	1,548.8	145.51	1,694.30
	Free f	0.509	0.0726	0.0258023–0.148	1,726.37	449.68	2,176.05
15 m versus 25 m	Full	0.978	0.00150	6.17×10^{-5}–0.00415	1,527.93	160.83	1,688.76
	$f = 0$	–	0.000892	4.42×10^{-5}–0.00251	1,528.31	163.07	1,691.38
	$\theta^{II} = 0$	0.978	–	–	1,545.80	144.02	1,689.83
	Free f	0.4992	0.0696	0.0233–0.144	1,720.85	448.87	2,169.72

Estimates of f, θ^{II} and the 95% credibility interval for θ^{II} (see "Methods" for details) are shown for each of four different models tested. Dbar is a measure of how well a model fits the data (models with smaller values are preferred) and pD indicates the effective number of parameters being estimated. The deviant information criterion (DIC) is a model-choice criterion that takes into account both of the above. Models with smaller DIC values are preferred, but a difference of more than six units is required to indicate strong support for one model over another. Results are shown first for the comparison between the intertidal and subtidal morphotypes, then among the four populations sampled from different depths, and finally for each of the different pairwise population comparisons

Adaptive strategies in marine gastropods

Gastropods inhabiting marine rocky shores provide excellent model systems with which to study the genetic and phenotypic basis of adaptation, partly due to their low vagility in the face of strong selection gradients imposed by a highly heterogeneous environment (Butlin et al. 2008). Two broad classes of adaptive strategy prevail (Johannesson 2003); the first of these is a 'generalist' strategy which combines a phenotype that fits most common habitats with dispersal through long-lived planktonic larvae. These larvae are able to rapidly colonise new sites, but because recruitment occurs from a common gene pool there appears to be little opportunity for local genetic adaptation. In contrast, direct-developing species adopt a 'specialist' strategy which lacks an effective dispersal stage, thereby preventing rapid colonisation but promoting local adaptation, which may in turn generate additional barriers to gene exchange in

the form of partial reproductive isolation. *N. concinna* appears to be a classic example of a generalist, occupying a broad range of habitats across the maritime Antarctic region (Powell 1951) and being a broadcast-spawner with pelagic larvae that can survive for up to 2 months in the water column (Bowden et al. 2006). Consequently, De Aranzamendi et al.'s (2008) claim that the intertidal and subtidal morphotypes of this species are genetically well-differentiated represents a significant departure from classical theory. To explore this further, we therefore collected additional empirical data from limpets at Adelaide Island, Antarctica.

Patterns of morphological variation

Prior to genetic analysis, we sought to confirm that previously reported morphological differences between the two *N. concinna* morphotypes (e.g. Walker 1972; Nolan 1991; de Aranzamendi et al. 2008) were present within our study population. As anticipated, the shells of intertidal limpets were both heavier and thicker than those of subtidal limpets. This difference probably relates to the fact that the intertidal zone is a far more physically stressful environment, with animals experiencing greater exposure to the impact of incidental hard objects (saltating clasts), crushing by sea-ice and pecking by gulls (Shabica 1976; Nolan 1991). However, the absence of subtidal engulfing predators (e.g. starfish, nemerteans and anemones) may also be important. As expected, shells of the intertidal morphotype were also found to be taller. This difference appears to reflect adaptation to desiccation and heat stress, with taller shells having a relatively small area of contact with the substratum that reduces water loss by evaporation (Vermeij 1973) and a larger shell surface area that facilitates heat loss by conduction. A recent meta-analysis by Harley et al. (2008) suggests that the latter effect is remarkably strong, with the ratio of shell height to length explaining over 90% of the variation in maximum predicted body temperature across a wide range of limpet species.

We were initially surprised to find significant differences between the two morphotypes in the ratio of shell breadth to length because these have not been universally reported. However, further exploration using Fourier analysis revealed a subtle pattern in which subtidal shells were relatively pear-shaped and intertidal shells more quadrangular in outline. An identical pattern has previously been described in *N. concinna* (Nolan 1991) as well as in other patellid species (Hockey and Branch 1983) using anterior to posterior breadth ratios (or 'shell uniformity indices') and has been shown to be independent of shell size. According to these authors, such a pattern could result from selective predation by birds such as the Dominican gull *Larus dominicanus*, with pear-shaped individuals having a recognisable anterior and posterior that makes them more vulnerable to attack. However, differences in shell outline could also be related to different hydrological conditions in intertidal and subtidal habitats, with a more elongated, elliptical form offering less resistance to wave action than a pear-shaped shell (Branch and Marsh 1978). The functional significance of these different shell forms could also be associated with differences in foot size and shape which are known to affect both the speed of locomotion and strength of adhesion to the substratum (Miller 1974).

Since the ability to right when disturbed has been shown to differ between limpets from 6 m and 25 m depths (S. Morley, personal communication), we not only compared the intertidal and subtidal morphotypes but also examined a more extensive depth profile. Significant differences in shell mass, thickness and height were found among the subtidal populations with, for example, shells from 6 m depth being significantly heavier (ANOVA, $F_{1,190} = 25.7$, $P < 0.0001$) thicker (ANOVA, $F_{1,190} = 73.37$, $P < 0.0001$) and taller (ANOVA, $F_{1,190} = 23.94$, $P < 0.0001$) than those from 25 m depth. This finding suggests that shell shape differences in *N. concinna* are not driven simply by factors that are specific to the intertidal zone. To date, five main factors have been proposed to account for the differences in shell shape between intertidal and subtidal limpets: (1) physiological responses to aerial exposure (Weihe and Abele 2008); (2) intertidal predation primarily by birds (Nolan 1991); (3) heat stress and desiccation (Vermeij 1973); (4) resistance to freezing and encasement in ice (Waller et al. 2006) and (5) resistance to physical disturbance by ice (Shabica 1976). We have shown that trends in shell thickness and the ratio of height to length are continuous down to 25 m depth, suggesting that although factors specific to the intertidal zone may play a role in generating some of the observed morphological differences, at least one other factor must operate over the entire depth range. A plausible candidate is ice disturbance, the frequency of which follows a similar pattern to that shown here for shell shape with a dramatic increase in frequency at shallow and intertidal depths over subtidal sites and a gradual decrease in frequency in the subtidal (Brown et al. 2004). Shape variation might also be due to differences in growth rates (Vermeij 1973), although this is difficult to test in our study because annual winter rings of the shells were too inconspicuous to allow for reliable age determination of the individuals and therefore, testing for allometric growth with age.

Patterns of genetic variation

The finding of continuous morphological variation along a depth cline is clearly at odds with a simple model of two discrete morphotypes comprising genetically divergent populations. In our genetic analysis, we therefore looked

not only for differentiation between the two putative morphotypes, but also among the four populations sampled from different depths. Despite using much the same analytical framework as de Aranzamendi et al. (2008), we found no evidence for genetic differences either between the two morphotypes or by depth. One possible reason for this discrepancy could relate to the specific populations examined, with de Aranzamendi et al. (2008) analysing three populations from Potter's Cove in the South Shetland islands, while our study population was located over 700 miles southwards towards the base of the Antarctic Peninsula. We collected samples from around Rothera for obvious practical reasons, with access to diving facilities enabling the collection of large sample sizes from several different depths, which would not have been possible at other more remote sites. Moreover, it is unclear why populations from Adelaide and Potter's Cove would differ in their propensity for the two morphotypes to be genetically divergent, particularly given that both sites show the same morphological variation, and a large unpublished AFLP dataset spanning the Antarctic Peninsula and including a population from Potter's Cove (Hoffman et al. MS in prepration) indicates that these populations are essentially panmictic.

Alternatively, the discrepancy could be linked to methodological differences between the two studies. For example, de Aranzamendi et al. (2008) analysed a total of only 108 individuals, and because these were distributed across three populations and three different depths, sample sizes for specific population: depth combinations were as small as 11 individuals. This could provide an explanation for why significant differences between the morphotypes were only found using F_{st} at the 'PC' population, which was represented by a sample size almost twice that of the other populations. To avoid this problem, we felt it important to obtain large and balanced sample sizes from each of the depths. Therefore, we opted to focus on a single population, collecting 96 samples from each of four different depths (total $n = 384$ individuals).

Another methodological difference between our study and that of de Aranzamendi et al. (2008) lies in the choice of genetic markers employed. De Aranzamendi et al.'s (2008) use of ISSRs is understandable because these markers are relatively easy to develop, facilitating rapid data collection. However, ISSRs tend to be less reliable than AFLPs in terms of reproducibility, robustness and the number of reported artefacts, (Meudt and Clarke 2007) making it particularly important to quantify the genotyping error rate. Unfortunately, although de Aranzamendi et al. (2008) state that their amplification patterns were highly repeatable, they did not report their error rate. In contrast, we independently re-extracted and re-genotyped over 10% of all samples ($n = 40$) to reveal an error rate of only 0.008 per reaction.

Is shell shape phenotypically plastic in Antarctic limpets?

Phenotypic plasticity has long been implicated as a strategy to cope with variable environments in many gastropod species characterised by planktonic larval dispersal (Kemp and Bertness 1984; deWolf et al. 1997; Parsons 1997), and a variety of limpet species are known to exhibit shell shape variation in relation to environmental factors such as exposure to water movement (Warburton 1976; Branch and Marsh 1978; Baxter 1983). Moreover, when Moore (1934) transplanted *Patella vulgata* individuals from an exposed shore to a site permanently covered by water, he found that the angle of the newly deposited shell material was far shallower after transplantation, indicating a more-or-less immediate transition to the flatter subtidal shell form. One mechanism that would account for such a pattern proposes that in desiccating environments, limpets clamp more tightly down onto the substrate, thereby pulling down the mantle such that if the mantle glands deposit the shell while in this position, the shell circumference will be reduced and the shell taller (Orton 1928, reviewed by Vermeij 1973).

Our results are clearly more consistent with an explanation of phenotypic plasticity than of two genetically divergent populations. However, a number of caveats should be born in mind. The first of these is a statistical issue: our inability to reject the hypothesis of population differentiation is strictly speaking not proof that there are no genetic differences between the morphotypes. Thus, it is possible that genetic differences between the two forms could exist but have gone undetected, perhaps due to low statistical power or limited resolving power of the markers used. However, our sample sizes were unusually large (96 individuals per population, total $n = 384$) and the number of loci that we used was also within the range recommended for studies of genetic structure (e.g. Cavers et al. 2005; Bonin et al. 2007). Moreover, de Aranzamendi et al. (2008) detected significant differences between the two morphotypes with much smaller sample sizes and many fewer loci. Therefore, if the differences that they reported were due to genome-wide divergence, our study should have been sufficiently powerful to easily detect a similar magnitude of effect if this were also present at Adelaide Island.

A second caveat is that the observed phenotypic differences need not necessarily reflect genome-wide divergence, but could instead be associated with one or a small number of loci. This is plausible given that even small numbers of pleiotropic genes can be responsible for rapid and extensive adaptive phenotypic change (e.g. Wagner et al. 2008). Unfortunately, little is currently known about pleiotropic effects in relation to shell shape in marine molluscs, although shell thickness and spire height were shown to be genetically correlated in *L. obtusata*, facilitating rapid and simultaneous changes in both of these traits in response to a

range expansion of predatory shore crabs (Seeley 1986). Moreover, studies using AFLPs have identified small numbers of loci that appear to be linked to phenotypic differences between intertidal and subtidal *L. saxatilis* morphotypes (Wilding et al. 2001; Galindo et al. 2009).

Finally, although previous authors have sought to attribute differences between the two *N. concinna* morphotypes to either genetic or plastic mechanisms (Beaumont and Wei 1991; de Aranzamendi et al. 2008), it is possible that both could be involved. This has been demonstrated in two direct-developing *Littorina* species. *L. saxatilis* and *L. obtusata*, suggesting that natural selection may act upon genetic variation in plastic traits (Janson 1982; Johannesson and Johannesson 1996; Trussell and Smith 2000). In the future, it might therefore be interesting to conduct transplantation experiments to determine whether shell shape variation in *N. concinna* is entirely plastic, or whether it also has a population-specific component.

Conclusion

Our study demonstrates that populations of *N. concinna* sampled from different depths at Adelaide Island are panmictic with respect to a large panel of AFLP markers. This finding is consistent with an explanation of phenotypic plasticity, although we are unable to discount the possible involvement of one or a small number of genes.

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References

Ajmone-Marsan J, Valentini A, Cassandro M, Vecchiotti-Antaldi G, Bertoni G, Kuiper M (1997) $AFLP_{TM}$ markers for DNA fingerprinting in cattle. Anim Genet 28:418–426

Baxter JM (1983) Allometric relationships of *Patella vulgata* L. Shell characters at three adjacent sites at Sandwick Bay in Orkney. J Nat Hist 17:743–755

Beaumont AR, Wei JHC (1991) Morphological and genetic variation in the Antarctic Limpet *Nacella concinna* (Strebel, 1908). J Molluscan Stud 57:443–450

Bensch S, Akesson M (2005) Ten years of AFLP in ecology and evolution: why so few animals? Mol Ecol 14:2899–2914

Bonin A, Bellemain E, Bronken Eidesen P, Pompanon F, Brochmann C, Taberlet P (2004) How to track and assess genotyping errors in population genetic studies. Mol Ecol 13:3261–3273

Bonin A, Ehrich D, Manel S (2007) Statistical analysis of amplified fragment length polymorphism data: a toolbox for molecular ecologists and evolutionists. Mol Ecol 16:3737–3758

Bowden DA, Clarke A, Peck LS, Barnes DKA (2006) Antarctic sessile marine benthos: colonisation and growth on artificial substrata over 3 years. Mar Ecol Prog Ser 316:1–16

Branch GM, Marsh AC (1978) Tenacity and shell shape in six Patella species: adaptive features. J Exp Mar Biol Ecol 34:111–130

Brown KM, Fraser KPP, Barnes DKA, Peck LS (2004) Ice scour frequency dictates Antarctic shallow-water community structure. Oecologia 141:121–129

Butlin RK, Galindo J, Grahame JW (2008) Sympatric, parapatric or allopatric: the most important way to clasify speciation? Philos Trans R Soc Lond B Biol Sci 363:2997–3007

Caballero A, Quesada H, Rolán-Alvarez E (2008) Impact of AFLP fragment size homoplasy on the estimation of population genetic diversity and the detection of selective loci. Genetics 179:539–554

Cavers S, Degen B, Caron H, Lemes MR, Margis R, Salgueiro F, Lowe AJ (2005) Optimal sampling strategy for estimation of spatial genetic structure in tree populations. Heredity 95:281–289

Crampton JS, Haines AJ (1996) Users' manual for programs HANGLE, HMATCH and HCURVE for the Fourier shape analysis of two-dimensional outlines. Institute of Geological and Nuclear Sciences Science Report 96. 37:1–28

Crampton JS, Maxwell PA (2000) Size: all it's shaped up to be? Evolution of shape through the lifespan of the Cenozoic bivalve Spissatella (Crassatellidae). In: Harper EM, Taylor JD, Crame JA (eds) The evolutionary biology of the Bivalvia. The Geological Society of London, London, pp 399–423

Crawley MJ (2002) Statistical computing, an introduction to data analysis using S-plus. Wiley, Chichester

Dasmahapatra KK, Hoffman JI, Amos W (2009) Pinniped phylogenetic relationships inferred using AFLP markers. Heredity 103:168–177

de Aranzamendi MC, Sahade R, Tatian M, Chiappero MB (2008) Genetic differentiation between morphotypes in the Antarctic limpet *Nacella concinna* as revealed by inter-simple sequence repeat markers. Mar Biol 154:875–885

de Wolf H, Backeljau T, Madeiros R, Verhagen R (1997) Microgeographical shell variation in *Littorina striata*, a planktonic developing periwinkle. Mar Biol 129:331–342

de Wolf H, Backeljau T, Verhagen R (1998a) Congruence between allozyme and RAPD data in assessing macrogeographical genetic variation in the periwinkle *Littorina striata* (Mollusca, Gastropoda). Heredity 81:486–492

de Wolf H, Backeljau T, Verhagen R (1998b) Spatio-temporal genetic structure and gene flow between two distinct shell morphs of the planktonic developing periwinkle *Littorina striata* (Mollusca: Prosobranchia). Mar Ecol Prog Ser 163:155–163

Dyer AT, Leonard KJ (2000) Contamination, error, and nonspecific molecular tools. Phytopathology 90:565–567

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567–1587

Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Notes 7:574–578

Galindo J, Morán P, Rolán-Alvarez E (2009) Comparing geographical genetic differentiation between candidate and non candidate loci for adaptation strengthens support for parallel ecological divergence in the marine snail *Littorina saxatilis*. Mol Ecol 18:919–930

Haines AJ, Crampton JS (2000) Improvements to the method of Fourier shape analysis as applied in morphometric studies. Palaeontology 43:765–783

Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4:4–9

Harley CDG, Denny MW, Mach KJ, Miller LP (2008) Thermal stress and morphological adaptations in limpets. Funct Ecol. doi:10.1111/j.1365-2435.2008.01496.x

Hockey PAR, Branch GM (1983) Do oystercatchers influence limpet shell shape? Veliger 26:139–141

Hoffman JI, Amos W (2005) Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. Mol Ecol 14:599–612

Hollander J, Lindergarth M, Johannesson K (2005) Local adaptation but not geographical separation promotes assortative mating in a snail. Anim Behav 70:1209–1219

Holsinger KE, Wallace LE (2004) Bayesian approaches for the analysis of population genetic structure: an example from *Platanthera leucophaea* (Orchidaceae). Mol Ecol 13:887–894

Holsinger KE, Lewis PO, Dey DK (2002) A bayesian approach to inferring population structure from dominant markers. Mol Ecol 11:1157–1164

Janson K (1982) Genetic and environmental effects on the growth rate of *Littorina saxatilis*. Mar Biol 69:73–78

Johannesson K (2003) Evolution in *Littorina*: ecology matters. J Sea Res 49:107–117

Johannesson B, Johannesson K (1996) Population differences in behaviour and morphology in the snail *Littorina saxatilis*: phenotypic plasticity or genetic differentiation? J Zool 240:475–493

Kaiser HF (1960) The application of electronic computers to factor analysis. Educ Psychol Measur 20:141–151

Kemp P, Bertness MD (1984) Snail shape and growth rates: evidence for plastic shell allometry in *Littorina littorea*. Proc Natl Acad Sci USA 81:811–813

Krauss SL (2000) Accurate gene diversity estimates from amplified fragment length polymorphism (AFLP) markers. Mol Ecol 9:1241–1245

Latch EK, Dharmarajan G, Glaubitz JC, Rhodes OE Jr (2006) Relative performance of Bayesian clustering software for inferring population substructure and individual assignment at low levels of population differentiation. Conserv Genet 7:295–302

Lynch M, Milligan BG (1994) Analysis of population genetic structure with RAPD markers. Mol Ecol 3:91–99

Maughan PJ, Saghai Maroof MA, Buss GR, Huestis GM (1996) Amplified fragment length polymorphism (AFLP) in soybean: species diversity, inheritance, and near-isogenic line analysis. Theor Appl Genet 93:392–401

Menge BA, Branch GM (2001) Rocky intertidal communities. In: Bertness MD, Gaines SD, Hay ME (eds) Marine Community Ecology. Sinauer Associates, Sunderland, pp 221–251

Meudt HM, Clarke AC (2007) Almost forgotten or latest practice? AFLP applications, analyses and advances. Trends Plant Sci 12:106–108

Miller SL (1974) Adaptive design of locomotion and foot form in prosobranch gastropods. J Exp Mar Biol Ecol 14:99–156

Moore HB (1934) The relation of shell growth to environment in *Patella vulgata*. Proc Malacological Soc London 21:217–222

Mueller UG, Wolfenbarger LL (1999) AFLP genotyping and fingerprinting. Trends Ecol Evol 14:389–394

Nolan CP (1991) Size, shape and shell morphology in the Antarctic Limpet *Nacella concinna* at Signy island, South Orkney islands. J Molluscan Stud 57:225–238

Orton JH (1928) Observations on *Patella vulgata*. II. Rate of growth of shell. J Mar Biol Ass UK 15:663–674

Palumbi SR (1999) All males are not created equal: fertility differences depend on gamete recognition polymorphisms in sea urchins. Proc Natl Acad Sci USA 96:12632–12637

Panova M, Hollander J, Johannesson K (2006) Site-specific divergencein parallel hybrid zones suggests nonallopatric evolution of reproductive barriers. Mol Ecol 15:4021–4031

Parsons KE (1997) Role of dispersal ability in the phenotypic differentiation and plasticity of two marine gastropods. Oecologia 110:461–471

Peck LS, Convey P, Barnes DKA (2006) Environmental constraints on life histories in Antarctic ecosystems: tempos, timings and predictability. Biol Rev 81:75–109

Picken GB (1980) Distribution, growth, and reproduction of the Antarctic Limpet *Nacella* (*Patinigera*) *concinna* (Strebel, 1908). J Exp Mar Biol Ecol 42:71–85

Pickles AR, Grahame J (1999) Mate choice in divergent morphs of the gastropod mollusc *Littorina saxatilis* (Olivi): speciation in action? Anim Behav 58:181–184

Polisky B, Greene P, Garfin DE, McCarthy BJ, Goodman HM, Boyer HW (1975) Specificity of substrate recognition by the EcoRI restriction endonuclease. Proc Natl Acad Sci USA 72:3310–3314

Powell AWB (1951) Antarctic and subanctarctic Mollusca: pelecypoda and gastropoda. Discov Rep (USA) 26:49–196

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

R Development Team (2005) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Rasband W (2008) http://rsbweb.nih.gov/ij, National Institute of Health, USA

Rolán-Alvarez E, Erlandsson J, Johannesson K, Cruz R (1999) Mechanisms of incomplete prezygotic isolation in an intertidal snail; testing behavioural models in wild populations. J Evol Biol 12:879–890

Rolán-Alvarez E, Carballo M, Galindo J, Morán P, Fernandez B, Caballero A, Cruz R, Boulding EG, Johannesson K (2004) Non-allopatric and parallel origin of local reproductive barriers between two snail morphotypes. Mol Ecol 13:3415–3424

Seeley RH (1986) Intense natural selection caused a rapid morphological transition in a living marine snail. Proc Natl Acad Sci USA 83:6897–6901

Shabica SV (1976) The natural history of the Antarctic limpet *Patinigera polaris* (Humbron & Jacquinot)

Stanwell-Smith D, Clarke A (1998) The timing of reproduction in the Antarctic limpet *Nacella concinna* (Strebel, 1908) (Patellidae) at Signy island, in relation to environmental variables. J Molluscan Stud 64:123–127

Strebel H (1908) Dei Gastropoden. *Wissenschaftliche Ergebnisse der Schwedischen Südpolar-Expedition*, 1901–1903. 6:1–112

Swanson WJ, Aquadro CF, Vacquier VD (2001) Polymorphism in abalone fertilization proteins is consistent with the neutral evolution of the egg's receptor for lysin (VERL) and positive Darwinian selection of sperm lysin. Mol Biol Evol 18:376–383

Trussell GC, Smith LD (2000) Induced defenses in response to an invading crab predator: an explanation of historical and geographic phenotypic change. Proc Natl Acad Sci USA 97:2123–2127

Vekemans X (2002) AFLP-SURV version 1.0. Distributed by the author. Laboratoire de Génétique et Ecologie Végétale. Université Libre de Bruxelles, Belgium

Vermeij GJ (1973) Morphological patterns in high-intertidal gastropods—adaptive strategies and their limitations. Mar Biol 20:319–346

Vos P, Hogers R, Bleker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Wagner GP, Kenney-Hunt JP, Pavlicev M, Peck JR, Waxman D, Cheverud JM (2008) Pleiotropic scaling of gene effects and the 'cost of complexity'. Nature 452:470–473

Walker AJM (1972) Introduction to the ecology of the Antarctic limpet *Patinigera polaris* (Hombron and Jaquinot) at Signy island, South Orkney islands. Br Antarctic Surv Bull 28:49–71

Waller CL, Worland MR, Convey P, Barnes DKA (2006) Ecophysiological strategies of Antarctic intertidal invertebrates faced with freezing stress. Polar Biol 29:1077–1083

Warburton K (1976) Shell form, behaviour, and tolerance to water movement in the limpet Patina pellucida (L.)(Gastropoda: Prosobranchia). J Exp Marine Biol Ecol 23:307–325

Weihe E, Abele D (2008) Differences in the physiological response of inter- and subtidal Antarctic limpets *Nacella concinna* to aerial exposure. Aquatic Biol 4:155–166

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370

Wilding CS, Butlin RK, Grahame J (2001) Differential gene exchange between parapatric morphs of *Littorina saxatilis* detected using AFLP markers. J Exp Biol 14:611–619

Zhivotovsky LA (1999) Estimating population structure in diploids with multilocus dominant DNA markers. Mol Ecol 8:907–913