

# GENETIC CONFIRMATION THAT INTERTIDAL AND SUBTIDAL MORPHS OF *PATELLA ULYSSIPONENSIS ASPERA* RÖDING (MOLLUSCA: GASTROPODA: PATELLIDAE) ARE CONSPECIFIC

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## ARQUIPÉLAGO



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The heavily exploited limpet species *Patella ulyssiponensis aspera* Röding (more widely known as *P. aspera* Röding) exhibits a largely discontinuous distribution in the Azores, occurring in the intertidal and subtidal zones. As a result two "habitat" morphs can be distinguished with respect to shell morphology. This paper examines the morphological and genetic variation of the two forms. Subtidal specimens were found to possess, on average, significantly larger shells but shorter radulae than intertidal specimens. Despite these marked morphological differences, very low levels of genetic divergence ( $I = 0.996$ ) were found between the two populations. This suggests that the two habitat morphs are conspecific and should be regarded as one breeding unit. Possible explanations of the bimodal distribution of this species in the Azores are considered.

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*Patella ulyssiponensis aspera* Röding (mais conhecida por *P. aspera* Röding), espécie de lapa intensamente explorada nos Açores, apresenta uma distribuição descontínua, ocorrendo nas zonas intertidal e subtidal. Como tal, esta espécie exibe duas formas ecológicas que podem ser distinguidas pela morfologia da concha. Este trabalho analisa a variação morfológica e genética destas duas formas. Os espécimes subtidais são caracterizados por conchas significativamente maiores e rádulas menores que os intertidais, contrastando com o baixo nível de divergência genética ( $I = 0.996$ ) encontrado entre as duas populações. Os resultados sugerem que as formas ecológicas são conspecíficas e devem ser reconhecidas como um único "stock". Algumas considerações são abordadas quanto à distribuição bimodal desta espécie nos Açores.

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## INTRODUCTION

Limpets of the genus *Patella* (L.) play a key role in the structuring of littoral communities. Their grazing strongly influences algal composition and diversity (BRANCH 1981; HAWKINS & HARTNOLL 1983), particularly in the northeast Atlantic (HAWKINS & al. 1992). Limpets are commercially valuable in the Azores and as such, in 1985, *Patella* species constituted the sixth most important fishery in São Miguel (MARTINS & al. 1987). Unfortunately,

the limpet stocks of the Azores have been declining steadily as a result of over-exploitation (MARTINS & al. 1987; MENEZES 1991). In 1983 the regional government financed the first stock assessment studies of limpets, leading to a ban on limpet collecting proclaimed in the Central and Eastern Island groups in 1989. In 1990 collecting was allowed in the Western group but only for non-commercial purposes.

There is still uncertainty regarding the taxonomy of the genus in the Azores. Although early taxonomists (DROUËT 1858; DAUTZENBERG 1889) considered that there were eight *Patella* species in

the Azores, this number has been reduced to two in the latest revision of the genus (CHRISTIAENS 1973; see also HAWKINS & al. 1990a). These two species are *P. ulyssiponensis* Gmelin, represented by the subspecies *P. u. aspera*, and *P. candei* d'Orbigny.

This study will examine one of the limpets identified by CHRISTIAENS (1973) as *P. ulyssiponensis aspera* and which the Azorians commonly call "lapa brava". It is a species found throughout the northeast Atlantic from southern Norway to the Mediterranean and through the Macaronesian Islands (PURCHON 1968; CHRISTIAENS 1973). It is generally found at low water on the shore, but in the Azores it occurs intertidally and subtidally down to depths of 10 m (HAWKINS & al. 1990a, b). Its distribution is not usually continuous, but interrupted by an algal turf in the lower eulittoral/shallow sublittoral (HAWKINS & al. 1990b). As a result two "habitat" morphs can be distinguished with respect to shell morphology. Intertidal specimens have smaller shells with thin, but marked, rays while subtidal specimens have larger shells with thicker, more pronounced ridges and small crenulations along the rim where the pallial tentacles protrude (HAWKINS & al. 1990a). Foot colour of limpets in both habitats can vary from yellow to bright orange or pink. Pallial tentacles are chalky white often with a black tinge surrounding the pallial margin (CHRISTIAENS 1973; HAWKINS & al. 1990a).

According to MARTINS & al. (1987), *P. u. aspera* in the Azores has been the limpet species more vulnerable to exploitation having suffered severely from both recruitment and growth overfishing. It is therefore important to determine whether the two habitat morphs represent two gene pools and are, hence, reproductively isolated, or whether the morphological differences are not correlated with genetic divergence. If the two morphs are genetically isolated, then different management strategies will be needed to manage the intertidal and subtidal populations.

Therefore, the aim of this study was to examine morphological and genetical variation of intertidal and subtidal forms of "lapa brava" which occur on the same shore. Variation in shell shape was quantified and differences in radular morphology were described in relation to the zonation patterns exhibited by the two forms. Enzyme elec-

trophoresis was used to investigate the level of genetic variation between forms to determine the integrity of the stock of this species. This technique has been used successfully in the past to discriminate cryptic species, establish levels of intraspecific variation and identify stocks for management purposes (see reviews by AVISE 1974; THORPE 1982; SMITH & al. 1990).

## MATERIAL AND METHODS

### Collection of samples

Specimens of *P. ulyssiponensis aspera* were collected in Monte da Guia, Horta, Faial in August, 1989 during the "Expedition Azores 1989". For details about this expedition see MARTINS & al. (1992). A total of 92 individuals were sampled intertidally from the sublittoral fringe and 98 subtidally (down to approximately 6 m) by snorkel diving. All specimens were transported live in insulated boxes to the Isle of Man and maintained in a seawater flow-through system in the laboratory before processing for electrophoresis.

### Morphometric analysis

Quantitative measurements of shell length (greatest distance between anterior and posterior ends), shell width (greatest distance perpendicular to the anterior-posterior axis), shell height (greatest vertical distance from the apex of the shell to the plane of the aperture) and radular length were taken to the nearest 0.5 mm using vernier calipers (see BEAUMONT & WEI 1991). The radula was dissected from each live individual by cutting through the top of the lip with a scalpel and removing the entire radular ribbon intact with forceps.

The results obtained were analysed by univariate and multivariate statistical methods. Simple Model I linear regressions (SOKAL & ROHLF 1981) were calculated for radular length against shell length for each population. The linear regressions were compared by analysis of covariance (SAS computer software package for the IBM, Release 6.03-GLM Procedure) to test for homogeneity of slopes and intercepts.



Three multivariate techniques were employed to examine the intricate relationships between the variables measured. Principal Component Analysis (PCA) was carried out (SAS-PCA Procedure) with the aim of ordering the variables in a small number of dimensions emphasizing the major patterns of variation between them (c.f. BLACKITH & REYMENT 1971; ANDERSON 1984). In this study PCA was based on log-transformed variables and the correlation matrix, providing maximal separation of the groups (JANSON & SUNDBERG 1983). Visual interpretation was facilitated by plotting principal components against each other (SAS-PROC PLOT PRINCOMP).

Once PCA was used to detect any "natural" multiple groups within the data set, canonical variate analysis (CVA) was performed (SAS-CVA Procedure) to find the characters responsible for the groupings (SUNDBERG 1988). This method maximizes differences between already defined groups, by producing linear combinations of the variables that have greatest between-group relative to within-group variability. Within-group variation is equally represented in all directions in canonical variate space (SNEATH & SOKAL 1973). The distance between each group centroid is measured with Mahalanobis  $D^2$  (SUNDBERG 1988). Variables contributing to group separation are determined by the relative magnitudes of the standardized canonical variate coefficients (REYMENT & al. 1984).

Once the data had been grouped and centroids calculated, discriminant function analysis (SAS-DISC Procedure) was applied. This procedure computes linear/quadratic functions for classifying observations into two or more groups based on the variables used. A set of discriminant functions is produced by which a specimen is allotted to one of the groups on which the analysis is based. To evaluate group differences, the proportion of correctly identified specimens in each group is computed (JANSON & SUNDBERG 1983). The ultimate purpose of this analysis is to minimize the probability of wrong assignment of unknown individuals (SNEATH & SOKAL 1973).

#### Radular structure analysis

The radulae of specimens from each population were examined using the scanning electron micro-

scope (S.E.M.). In order to eliminate tissue residues, each radula examined was prepared by removing it intact from the live animal, soaking it in 0.1M NaOH for approximately 90 minutes, and rinsing it in running tap water. Each radula was preserved in 70% alcohol until used for S.E.M.

Before coating the stub, each radula was air dried and mounted on the stub by using double-sided cellotape. The radula was mounted teeth upwards across the diameter of the stub. Each stub was placed in a sputter coater and coated with 60% gold/palladium. Each was viewed with a Philips 501B S.E.M using an accelerated voltage of 7.2 KV. Because of possible variations in tooth structure, degree of wear and mineralization along the length of the radula, photographs were taken of the anterior mid section in all specimens.

#### Electrophoretic analysis

Allozyme electrophoresis was carried out using standard horizontal starch gel techniques (see HARRIS & HOPKINSON 1978; RICHARDSON & al. 1986). Only healthy animals were used in the analysis. For each specimen the foot muscle was dissected whilst still alive and frozen rapidly by immersion in liquid nitrogen. It was stored at  $-20^{\circ}\text{C}$  until required for electrophoresis. Gels were prepared with 12.5% starch (Sigma Chemicals, Poole, Dorset). Tris-citrate pH 8.0 (WARD & BEARDMORE 1977) was the standard buffer system used, run at 120V (@5W) for seven hours. Staining methods followed those of SCHAAL & ANDERSON (1974) and HARRIS & HOPKINSON (1978). Only polymorphic loci (with most common allele  $<0.95$  frequency) were selected for further study. Seven enzyme systems revealing eight loci were employed: Adenylate kinase (AK, E.C.N. 2.7.4.3), Aldolase (ALD, E.C.N. 4.1.2.13), Isocitrate dehydrogenase (IDH, E.C.N. 1.1.1.42), Mannose phosphate isomerase (MPI, E.C.N. 5.3.1.8), Nucleoside phosphorylase (NP, E.C.N. 2.4.2.1), Glucose phosphate isomerase (PGI, E.C.N. 5.3.1.9), and Phosphoglucosmutase (PGM, E.C.N. 2.7.5.1).

The analysis of electrophoretic data was carried out using the computer software package Biosys-1 Release 1.7 (SWOFFORD & SELANDER 1989). Allele frequencies, heterozygosity, poly-

morphism and mean number of alleles per locus were calculated for each population. Due to small sample sizes (SOKAL & ROHLF 1981), genotypic departures from Hardy-Weinberg expectations were tested by chi-square analysis with alleles pooled into three classes: A- homozygotes for the most common allele, B- heterozygotes for the most common allele, C- all other genotypes. Allele frequencies of populations were compared with heterogeneity chi-square and NEI's (1972) index of genetic similarity, I, was calculated.

## RESULTS

Figures 1 and 2 show linear Model I regressions fitted by least square means for radular length against shell length for intertidal and subtidal *P. u. aspera*. Both slopes differ significantly from zero (ANOVA  $p < 0.0001$ , Table 1). The  $r^2$  values differ for both populations being greater for the subtidal (0.871) than the intertidal (0.586), indicating a larger amount of variation unexplained by the regression equation in the intertidal population. Analysis of covariance revealed no difference ( $F = 0.01$ ,  $p < 0.98$ ) between slopes of both populations but a significant difference ( $F = 213.78$ ,  $p < 0.0001$ ) for the y-intercepts. This shows that the

subtidal individuals possess shorter radulae than intertidal ones.

Differences in shell shape were detected by the multivariate analysis. PCA (Table 2a, b) shows that PC1 accounts for 70% of the total variation and approximates an isometric size vector (HUMPHRIES & al. 1981; SOMERS 1986), due to almost equal loadings of all the variables used. PC2 which accounts for 22% of the variation separates the samples on the basis of radular length (Figure 3).

CVA results (Table 3) separate the two populations by significant differences ( $D^2 = 3.34$ ,  $p < 0.0001$ ) in radular length coupled with shell length. This confirms the regression analysis showing the subtidal population having a lower radular fraction (see FISCHER-PIETTE 1935) than the intertidal one. Discriminant function analysis (Table 4) clearly separates the two groups morphologically by correctly classifying 89% of the intertidal population and 99% of the subtidal population.

Figure 4 shows the radular morphology of the intertidal and subtidal forms. In both specimens the pluricuspid tooth is marked by four cusps, with the external being the smallest. The intertidal morph shows a more hook-like tooth arrangement while the subtidal morph shows spade-like teeth.

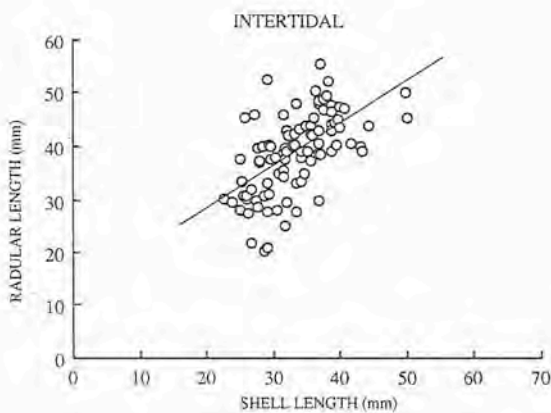


Fig. 1 - Linear regression of radular length (mm) against shell length (mm) in intertidal individuals of *P. u. aspera*.

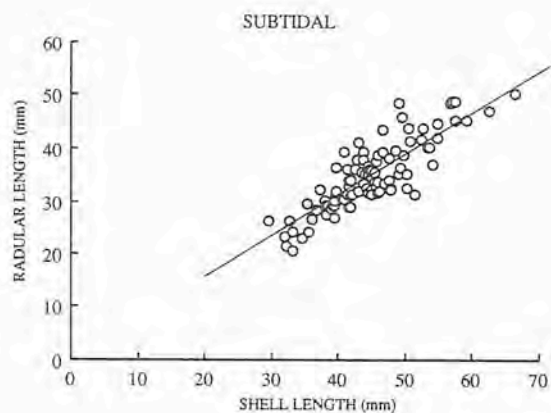


Fig. 2 - Linear regression of radular length (mm) against shell length (mm) in subtidal individuals of *P. u. aspera*.



Table 1  
Calculated relationships for radular length (mm) against shell length (mm) in two populations of *P. u. aspera*

	Regression equation	n	r <sup>2</sup>	p	Confidence interval (± 95%)
Intertidal	y = 0.798x + 12.214	92	0.586	<0.0001	0.116
Subtidal	y = 0.815x - 1.631	98	0.857	<0.0001	0.050

There is, however, no clear feature of the radular structure that can be used to distinguish intertidal from subtidal specimens.

Allele frequencies and sample sizes for both populations are given in Table 5. *Pgm-1* shows four alleles in both populations while *Ak* and *Idh-2* only show two. Both populations show the same mean number of alleles per locus (2.9) and the same levels of polymorphism (87.5%). Populations do differ in the proportion of observed and expected heterozygotes as measured by chi-square analysis (Table 6). The intertidal population

shows significant departures from Hardy-Weinberg equilibrium for all loci except *Mpi*. The subtidal population shows significant deviations for all loci. In both cases deviations are due to excesses of homozygotes (Table 6). Allele frequencies between populations do not differ significantly for any locus (Table 7) giving an overall chi-square value of 11.135,  $P < 0.74297$  for the comparison of all loci. This is reflected in the very high genetic identity (NEI 1972) value for the comparison of the two populations of 0.996, averaged for the eight polymorphic loci.

Table 2a

Summarised results of principal component analysis based on four morphometric characters of *P. u. aspera*. The variance of each component is expressed by the eigenvalue and the proportion of explained variation is indicated. The eigenvectors indicate how much each character influences the total variation of each component.

	Principal Component			
	1	2	3	4
Eigenvalue	2.80572	0.87236	0.28611	0.03580
α of variation	0.701430	0.218091	0.071528	0.008951
Eigenvectors				
Shell length	0.547509	-0.367868	0.287305	-0.694524
Shell width	0.545889	-0.394578	0.184617	0.715703
Shell height	0.520083	0.237656	-0.818573	-0.054507
Radular length	0.362979	0.807775	0.461855	0.049347

Table 2b

Correlation values of the four morphometric variables used for the principal component analysis

	Shell length	Shell width	Shell height	Radular length
Shell length	1.0			
Shell width	0.9626	1.0		
Shell height	0.6567	0.6701	1.0	
Radular length	0.3351	0.3036	0.5889	1.0

Table 3

Summarised results of canonical variate analysis based on four morphometric characters of intertidal and subtidal *P. u. aspera*, showing the extent that each character influences the canonical coefficient together with Mahalanobis ( $D^2$ ) generalised distance between group centroids

Character	Standardized Canonical Coefficient 1		Mahalanobis Distance	
			Intertidal	Subtidal
Shell length	1.6121	Int.	-	-
Shell width	0.3119			
Shell height	0.0871	Subt.	3.3430*	-
Radular length	-1.3017			

\*Significant at  $p < 0.001$  (F-test)

## DISCUSSION

There was marked morphological differentiation between intertidal and subtidal populations of *P. ulyssiponensis aspera* from Monte da Guia. Subtidal specimens have on average significant larger shells but shorter radulae. Despite this morphological variation, for no locus was any significant genetic difference detected between the two forms by electrophoresis. It must be noted that both populations show an unusually high proportion of homozygotes for most loci investigated.

Possible explanations for this phenomenon are being considered in a more detailed study of genetic variation of *P. u. aspera* throughout its geographical range (Corte-Real unpubl.). The high genetic identity value (NEI 1972) of 0.996 is within the range considered by several authors (i.e. 0.9-1.0, AYALA & al. 1974; THORPE 1982, 1983; NEI 1987) to be typical of conspecific comparisons. It should be noted that the use of only polymorphic loci will cause some bias in the I value since such loci will generally show increased genetic divergence (SKIBINSKI & WARD 1982). Thus over a random sample of both polymorphic and monomorphic loci the true I value is likely to be even higher. This high genetic identity might imply any of three things. It is possible that

Table 4

Proportion of correctly assigned intertidal and subtidal individuals of *P. u. aspera* into each *a priori* group on the basis of their discriminant functions

	Number of observations and % classified into zonation		
	Intertidal	Subtidal	Total
Intertidal	82	10	92
%	89.13	10.87	100
Subtidal	1	97	98
%	1.02	98.98	100
Total	83	107	190
%	43.68	56.32	100
Priors	0.5	0.5	

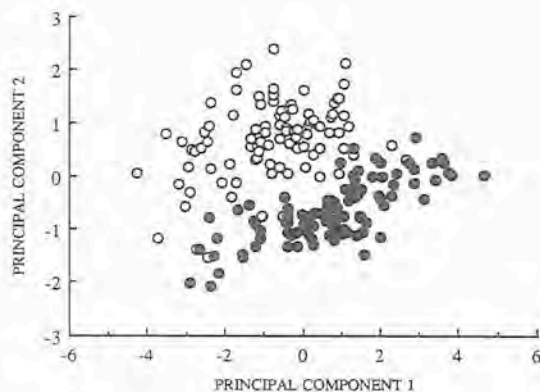


Fig. 3 - Scatter diagram of principal component 2 against principal component 1 in intertidal (o) and subtidal (•) individuals of *P. u. aspera*.



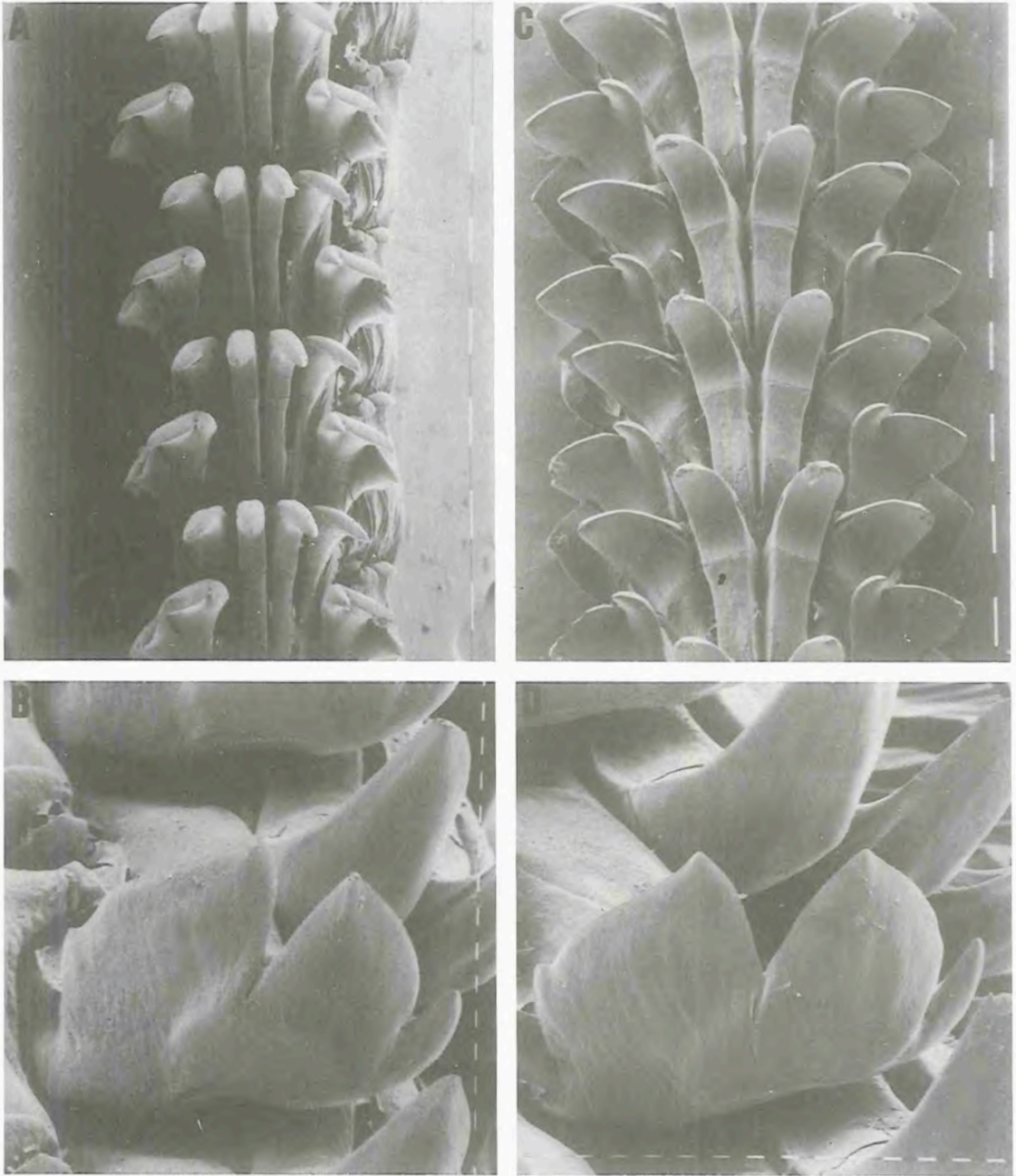


Fig. 4 - Radular morphology of intertidal (A, B) and subtidal (C, D) specimens of *P. u. aspera*. A and C give an overview of the radula showing the marginal, lateral and pluricuspid teeth (micron marker = 55.6  $\mu\text{m}$ ); B and D show the detail of the pluricuspid teeth (micron marker = 10  $\mu\text{m}$ ).

Table 5

Allele frequencies and sample sizes (N) for eight loci in intertidal and subtidal populations of *P. u. aspera*

LOCUS	INTERTIDAL	SUBTIDAL
<i>Ak</i> (N)	66	70
A	0.871	0.857
B	0.129	0.143
<i>Ald</i> (N)	56	54
A	0.134	0.120
B	0.586	0.481
C	0.277	0.398
<i>Idh-2</i> (N)	88	90
A	0.994	0.978
B	0.006	0.022
<i>Mpi</i> (N)	88	90
A	0.267	0.239
B	0.545	0.539
C	0.188	0.222
<i>Np</i> (N)	68	70
A	0.096	0.107
B	0.868	0.857
C	0.037	0.036
<i>Pgi</i> (N)	88	89
A	0.551	0.528
B	0.403	0.421
C	0.045	0.051
<i>Pgm-1</i> (N)	88	90
A	0.136	0.161
B	0.551	0.511
C	0.250	0.239
D	0.063	0.089
<i>Pgm-2</i> (N)	88	96
A	0.682	0.630
B	0.284	0.297
C	0.034	0.073

the small number of loci examined did not provide sufficient differentiation, as electrophoresis only reveals a proportion of the total genetic variation (FERGUSON 1980; NEI 1987) or, by chance, the loci employed were not the "correct" ones for detecting differences between the morphs. Alternatively, the two populations may constitute one panmictic unit with morphological variation being habitat-induced.

Several factors suggest that the latter might be the case despite significant excesses of homozy-

gotes. Firstly, there is much evidence suggesting that the morphology of limpets is heavily influenced by environmental conditions, in particular with respect to the major environmental gradients of exposure to wave action and tidal height (c.f. MOORE 1934; DAVIES 1969; SELLA 1976; NOLAN 1991), as well as by biological factors such as competition and density (BOWMAN & LEWIS 1977). Secondly, reduced radular lengths have been reported previously for sublittoral (SELLA 1976) or low shore specimens (BRIAN & OWEN 1952) compared with specimens higher in the intertidal zone. These differences could be due to the differential feeding habits of the two populations. Because limpets feed largely when submerged or in damp conditions (BRANCH 1981; HAWKINS & HARTNOLL 1983), sublittoral individuals spend longer feeding and therefore shorter radulae may result merely from increased wear (SELLA 1976). Thirdly, the lack of differentiation in the radular morphology between the two morphs—a feature which has been considered as diagnostic in *Patella* species (FISCHER-PIETTE 1935; FISCHER-PIETTE & GAILLARD 1959; CHRISTIAENS 1973; POWELL 1973), suggests the forms considered here are likely to be conspecific. Furthermore, the study of the reproductive patterns of the populations (MARTINS & al. 1987; MENEZES 1991) has shown that their breeding cycles are synchronised, although size at first sexual maturity differs between the morphs (MENEZES 1991). This would also render possible genetic exchange between the populations.

Similar findings have been reported from both Australia and Antarctica for other morphological and genetical studies of limpets exhibiting a comparable bimodal distribution (BLACK & JOHNSON 1981; NOLAN 1991; BEAUMONT & WEI 1991). In all cases, morphological differentiation was interpreted as a phenotypic response to the environment. If the same situation occurs in the Azores, it could be tested by transplants (see MOORE 1934).

It is interesting to speculate on the discontinuous distribution of *P. u. aspera* in the Azores (HAWKINS & al. 1990a). It is possible that limpets cannot extend their downshore range due to the ability of the algae to dominate space exceeding the grazing capability of limpets (BRANCH 1981; UNDERWOOD & JERNAKOFF 1981; HAWKINS & HARTNOLL 1983). This effect would be aggra-



Table 6

Deviations of observed genotype frequencies from Hardy-Weinberg expectations, measured by chi-square analysis with alleles pooled into three classes

LOCUS	CLASS	OBS. FREQ.	EXP. FREQ.	$\chi^2$	P
INTERTIDAL					
<i>Ald</i>	A	26	19.324	13.603	0.001
	B	14	27.351		
	C	16	9.324		
<i>Mpi</i>	A	29	26.057	1.601	0.206
	B	38	43.886		
	C	21	18.057		
<i>Np</i>	A	57	51.733	39.792	0.001
	B	4	15.733		
	C	7	1.133		
<i>Pgi</i>	A	43	26.606	49.920	0.001
	B	11	43.789		
	C	34	17.606		
<i>Pgm-1</i>	A	33	26.606	7.594	0.006
	B	31	43.789		
	C	24	17.606		
<i>Pgm-2</i>	A	51	40.800	25.210	0.001
	B	18	38.400		
	C	19	8.800		
SUBTIDAL					
<i>Ald</i>	A	21	12.393	22.016	0.001
	B	10	27.215		
	C	23	14.393		
<i>Mpi</i>	A	34	26.011	11.486	0.001
	B	29	44.978		
	C	27	19.011		
<i>Np</i>	A	56	51.367	21.094	0.001
	B	8	17.266		
	C	6	1.367		
<i>Pgi</i>	A	40	24.695	42.383	0.001
	B	14	44.610		
	C	35	19.695		
<i>Pgm-1</i>	A	31	23.385	10.318	0.001
	B	30	45.229		
	C	29	21.385		
<i>Pgm-2</i>	A	48	38.010	19.170	0.001
	B	25	44.979		
	C	23	13.010		

Table 7

Heterogeneity chi-square analysis for the comparison of allele frequencies of intertidal and subtidal populations of *P. u. aspera*

LOCUS	No. ALLELES	$\chi^2$	D.F.	P
<i>Ak</i>	2	0.114	1	0.73515
<i>Ald</i>	3	3.678	2	0.15895
<i>Idh-2</i>	2	1.758	1	0.18486
<i>Mpi</i>	3	0.809	2	0.66720
<i>Np</i>	3	0.102	2	0.95041
<i>Pgi</i>	3	0.204	2	0.90292
<i>Pgm-1</i>	4	1.497	3	0.68304
<i>Pgm-2</i>	3	2.972	2	0.22627
Totals		11.135	15	0.74297

vated by the sustained human exploitation of limpets creating open spaces on the rock, readily available for the colonizing of the algal turf (MARTINS & al. 1987).

The differential shell lengths of individuals from the two habitats might be due to several factors. The subtidal habitat in which they are most abundant (MARTINS & al. 1987; MENEZES 1991) might represent the optimal habitat of this species and this would be reflected in faster growth rates and larger sizes in this zone. Alternatively, the intertidal zone could be acting as a "nursery" ground for juveniles and younger adults with larger/older adults migrating into the subtidal. In both cases, this could only be tested by marking experiments and tracing movements of individuals.

It is therefore probable that the intertidal and subtidal forms of *P. u. aspera* represent one breeding unit and should be regarded as a single species in the Azores. The results of this study provide yet another example of the morphological plasticity of limpets resulting from their ability to colonise a variety of littoral habitats. This plasticity has confused generations of taxonomists!

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