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Morphological and genetic differentiation of *Patella granularis* (Gastropoda: Patellidae): recognition of two sibling species along the coast of southern Africa

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Abstract

Morphological and isozyme variations between 13 populations of the species hitherto named *Patella granularis* were investigated to see whether differences in shell structure between the west coast versus the south and east coasts of southern Africa are supported by other morphological features or by genetic differences. The shells showed a definite decrease in size from west to east, but this is correlated with productivity and is of no diagnostic use in distinguishing between populations. Discriminant functions analysis based on shell morphometrics failed to separate populations from the three coastal regions. Shells from the northern east coast do, however, have shell nodules with a dark pigmentation, distinctly separating them from those further south and west. No differences in radular or soft part morphology were detected between the populations, but the four northernmost populations on the east coast have a significantly shorter Z looping of the gut than the other populations along the coast. Significant microstructural differences in the sperm were also detected between these two groups of populations. Electrophoretic analysis of 16 enzyme loci failed to detect any significant differences between the west and south coast populations, but revealed a genetic identity (Nei) of 0.528 as well as four diagnostic alleles between the four northernmost populations from the east coast compared with those to the south and west. The two genetically distinct forms occurred sympatrically at one of the study sites on the east coast (Coffee Bay). It was concluded the two groupings were sufficiently different to warrant the recognition of a separate species, which is centred in KwaZulu-Natal on the east coast and extends south to Coffee Bay, from where it is replaced by *P. granularis*. There is, however, no evidence at all that the west coast populations are in any way separable from the remaining populations of *P. granularis* on the south coast.

Key words: patellid, limpet, allozymes, genetics, morphology

INTRODUCTION

The patellid limpet *Patella granularis* L., as currently recognized, is the most widely distributed of all the southern African limpet species. A generalist intertidal grazer (Branch, 1981), it ranges from Rocky Point in Namibia, in the cold, nutrient-rich waters of the Benguela upwelling system, through to the warmer waters of the south and east coasts, ostensibly extending as far east as Umpangazi (Stephenson, 1937; Koch, 1949; Kensley & Penrith, 1973; Powell, 1973). Throughout this range *P. granularis* shows variation in shell size and morphology (Koch, 1949; Kilburn & Rippey, 1982) corresponding with differences in the oceanic environment (Stephenson, 1937).

Various authors have reported variations in the structure and mineralogy of *P. granularis* shells from the coast of South Africa (Cohen, 1988; Cohen & Branch, 1992). The ratio of aragonite:calcite in its shells is correlated with sea temperature, with the west coast shells having a greater percentage of calcite than the south and east coast shells. Further differences between the west coast versus the south and east coast individuals have been found in the microstructure of the shell. The shell is deposited in discrete layers, which are numbered with reference to the myostracum (*m*): layers $m-1$ and $m-2$ lie inside the myostracum, whereas $m+1$, $m+2$ and $m+3$ lie sequentially outside it. Individuals to the west of Cape Point have shells with six discrete structural layers, whereas individuals east of Cape Point only have five layers (lacking the innermost calcitic $m-2$ layer) (MacClintock, 1967; Cohen & Branch, 1992). Secondly, the aragonitic $m+1$ layer of the south and east coast shells is well developed,

whereas shells from the west coast have a relatively thin $m+1$ layer, with a well-developed outer foliar $m+2$ layer (Cohen & Branch, 1992).

Molluscan taxonomy has historically been based purely on morphological characters, with shell form and radular structure being the most commonly used characters. However, the large degree of morphological plasticity exhibited by limpets has plagued the taxonomy of this group (Powell, 1973) and the value of these morphological characters is therefore questionable (Côrte-Real, Hawkins & Thorpe, 1996*b*). For example, ratios of shell proportions and radular length within the genus *Patella* reflect different habitats and consequent responses of individuals to local environmental stresses (Powell, 1973). Differences in size and shell morphology of *P. granularis*, and the fact that shells from the east coast also appear to be darker in appearance than those from the west coast (Kilburn & Rippey, 1982), has led to numerous uncertainties with regard to the taxonomy of this species in the past (see Powell, 1973). However, despite variation in shell structure, concordance in radular structure and anatomy along the coast (Koch, 1949) has led taxonomists to suggest that a single species is involved.

In the last few decades, the use of molecular techniques in taxonomic studies, often in conjunction with more traditional morphological approaches, has provided increased taxonomic clarity about relationships within molluscan groups. Although similar studies have been carried out on a variety of other marine molluscs (Colognola *et al.*, 1986; Heller & Dempster, 1991; Boulding, Buckland-Nicks & Van Alstyne, 1993), very little published work integrates morphological and genetic characters of patellid limpets (Côrte-Real, Hawkins & Thorpe, 1996*a*).

Given the contrast in shell structure between the west coast individuals and those of the south and east coasts, we initially hypothesized that the populations in these two regions are distinct. Thus, it was decided to reassess the taxonomic status of *P. granularis* along the coast of southern Africa by a combination of morphological and biochemical methodologies. Previous analyses of prosobranch gastropods have highlighted certain informative characters, such as shell microstructure, radular morphology, digestive, nervous and reproductive systems, and sperm structure, that can be used for taxonomic purposes (Houbrick, 1988; Lindberg, 1988; Jamieson, Hodgson & Bernard, 1991; Kool, 1993). However, some of these characters (such as the anatomy of the reproductive system) are not applicable in the study of patellid limpets because they have a simple reproductive system with no ducts and glands (Ridgway, 1994). Thus, in the present study, shell characters and radular morphology, sperm microstructure, and gut loop coiling were examined to test for differences between populations of *P. granularis* along the southern African coast. Protein gel electrophoresis was used for the first time on *P. granularis* to determine the degree of genetic differentiation among the different populations. In this way it was hoped to determine whether the west coast populations of *P. granularis* form a morphologically and/or genetically distinct group from the south and east coast populations.

MATERIALS AND METHODS

Collection

Specimens of *Patella granularis* were collected from intertidal rocky shores at Swakopmund (Swakop) and Luderitz (Lud) in the cool temperate Namibian biogeographic province; Groen River (Groen) and Kommetjie (Kom) in the cool temperate Benguela province of the west coast; Kalk Bay (KBay), Port Elizabeth (PE) and East London (EL) representing the warm temperate Agulhas province on the south coast; and Dwesa (Dwesa), Coffee Bay (CBay), Oslo Beach (Oslo), Green Point (GrnPt) and Mapelane (Map) in the subtropical Natal biogeographic province on the east coast of South Africa (Fig. 1). The 4 biogeographic provinces mentioned follow those proposed by Emmanuel *et al.* (1992), but for simplicity the 2 cool temperate provinces are hereafter combined and termed the 'west coast'. Individuals from each site were preserved in 70% alcohol. For genetic analyses limpets were stored in an ultra deep-freeze (at $-80\,^{\circ}C$) for later electrophoretic analysis.

Morphological analyses

Shell texture and pedal coloration

The pigmentation of the nodules on the costae (Fig. 2) was recorded for all sample sites. The number of costae on the shells (Fig. 2) was counted for 15 individuals from all but 2 of the sample sites. Shells from Swakopmund and Groen River were excluded from this analysis because the costae were not always clearly defined due to erosion, therefore making them difficult to score consistently. Differences between the populations were tested using analysis of variance (ANOVA) and Tukey's honestly significant difference test (Statistica for Windows Release 5.1, StatSoft Inc., 1996).

The number of the nodules on the costae was also scored. The nodules were counted on the left and right anterior-most and posterior-most costae (Fig. 2). Paired *t*-tests between the left and right anterior-most costae revealed no significant differences, therefore the data were pooled. For the same reason, counts for the left and right posterior-most costae were similarly pooled. Both sets of pooled data complied with the assumptions of ANOVA, therefore ANOVA and Tukey's honestly significant difference test were performed on untransformed data to test for any significant differences between the populations. The pigmentation of the underside and the side of the foot was also recorded from all the sample sites.

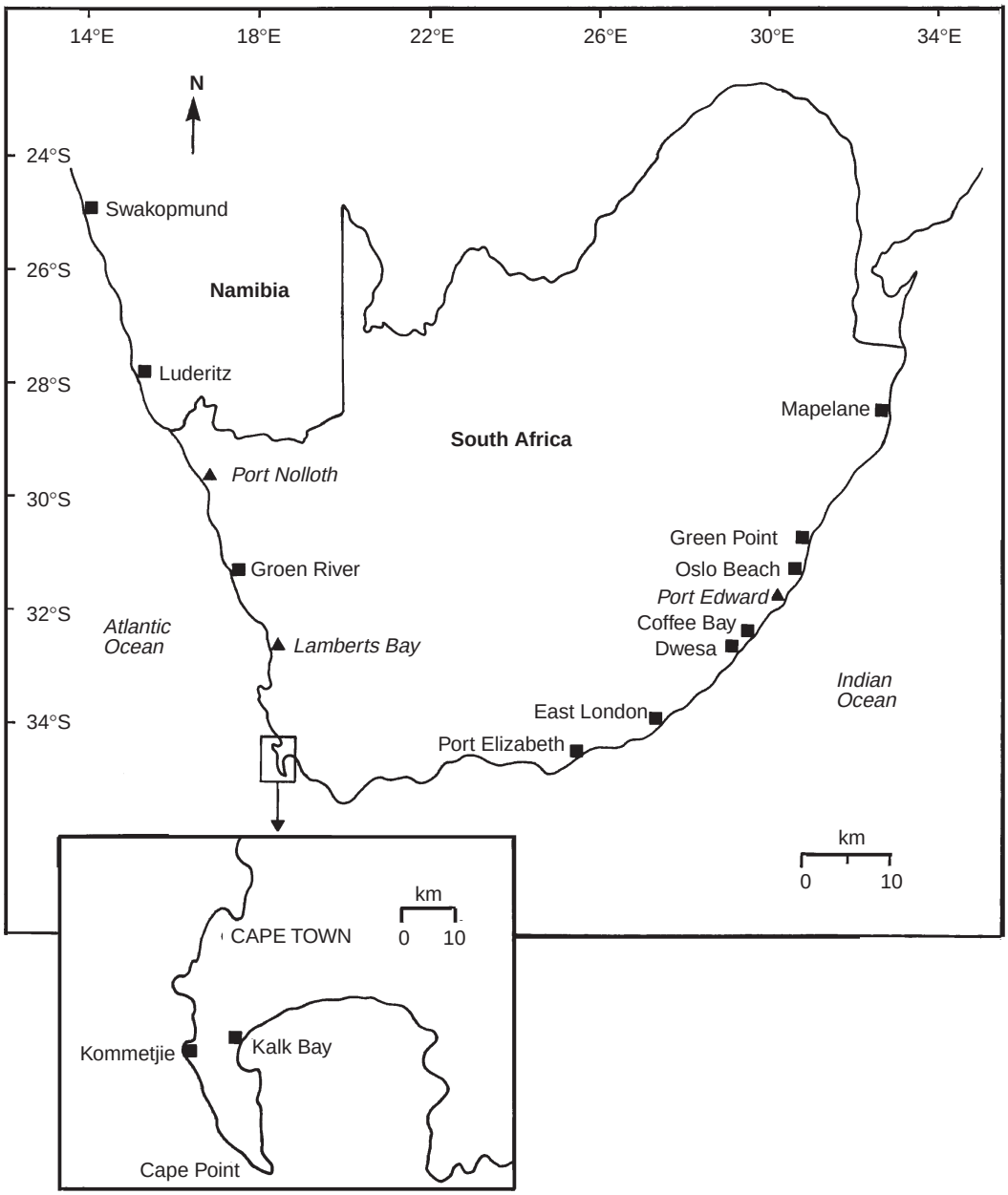

Fig. 1. Map of southern Africa showing the 12 sites (■) at which *Patella granularis* was sampled, supplemented with museum material from three sites (▲).

Shell morphometrics

Shell morphometrics were obtained by measuring the shells of 624 individuals from the 12 sample sites (Fig. 1), as well as from 3 additional sample sites from the dry mollusc collection at the South African Museum (SAM). These additional sites (also shown in Fig. 1) were Port Nolloth (SAM A52929) and Lamberts Bay (SAM A52930) from the west coast, and Port Edward (SAM A53165) from the east coast of South Africa. All shells used were in good condition with minimum erosion. Digital callipers (accuracy of 0.01 mm) were used to measure shell length (SL, greatest distance between anterior and posterior end), shell width (SW, greatest distance perpendicular to the anterior–posterior axis), and shell height (SH, greatest vertical distance from the apex of the shell to the plane of the aperture). Shell dry weight (WT) was measured and the internal shell volume (VOL) was determined from the weight of 70% alcohol which filled an upturned shell. Although morphometric ratios are widely employed in taxonomic studies, there are two problems with their use (Spivey, 1988). Firstly, ratios of original variates can change with an increase in size (Spivey, 1988), and secondly the use of ratios in regression analysis is flawed because the effect of size has not been removed from the shape variable (Atchley, Gaskins & Anderson, 1976), although ratios can be regressed on size and the effect of size

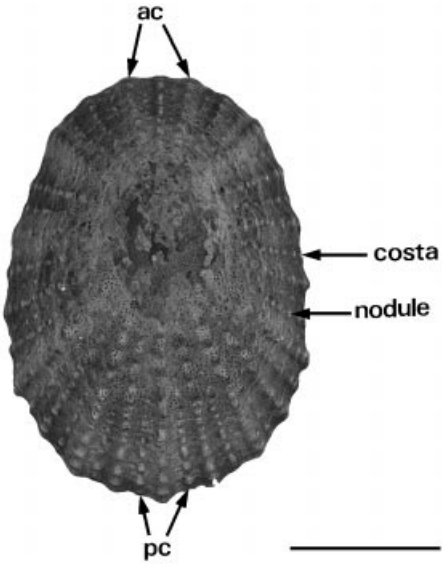

Fig. 2. Dorsal view of the shell of *Patella granularis* showing the costae, nodules and the costae on which the nodules were counted. ac, anterior costae, and pc, posterior costae, on which nodules were counted. Scale bar: 1 cm.

eliminated as a co-variate. It was thus decided not to use ratios but rather to use the original measurements for the multivariate statistics. Within each coastal region, no significant differences existed between the shell weight of the populations, so erosion was assumed to be uniform between the populations of the coastal regions and therefore shell dry weight was included in the analysis. The morphometric data were log transformed and analysed by stepwise discriminant functions analysis (Statistica for Windows Release 5.1, StatSoft Inc., 1996), which computes functions for classifying observations into 2 or more groups based on the variables used. A set of discriminant functions is produced by which a specimen is assigned to 1 of the groups on which the analysis is based (Côrte-Real *et al*., 1996*a*). The analysis was performed on all populations but for simplicity of presentation of the figure, the 16 populations were pooled into west, south, and east coastal regions.

Radular morphology

Radulae were dissected from 2 to 5 individuals from each of Groen River, Kommetjie, Kalk Bay, Port Elizabeth and Green Point and examined using scanning electron microscopy (SEM). Due to abrasion and variation in the degree of mineralization along the length of the radula, only the anterior mid-section of the radulae was examined. The extracted radulae were placed in a 10% solution of potassium hydroxide and ultrasonicated for 10 min. They were then transferred to 70% alcohol and ultrasonicated for a further 60 s, before dehydration through an alcohol series. The dehydrated radulae were then dried in a critical-point drier and mounted, teeth uppermost, on aluminium stubs, sputter-coated with gold-palladium and examined with a Cambridge S-200 Electron Microscope. For each radula, the number and arrangement of the lateral and marginal teeth, and the presence or absence of the rachidian tooth was recorded. Measurements were taken from the rachidian, the first lateral and the large pluricuspid tooth, the cusps (C) of which are numbered 1 to 4 from the outside to the centre of the radula. The following measurements (Fig. 3) were quantified: (a) ra/rb = ratio of the length of the cusp of the rachidian tooth relative to the length of the cusp of the adjacent lateral tooth, (b) C4a/C3a = ratio of the width of cusp 4 relative to the width of cusp 3, (c) C2a/C3a = ratio of the lower width of cusp 2 relative to the width of cusp 3, (d) C2a/C2c = ratio of lower width of cusp 2 relative to upper width of cusp 2 and (e) C2b/C3b = ratio of height of cusp 2 relative to height of cusp 3. Due to the small sample sizes, the data from the 5 populations were pooled into the 3 coastal zones outlined above. A Kruskal–Wallis test and the Wilcoxon 2-sample test were performed based on the methods outlined by Dunn (1964) to test for significant differences between the 3 regions for the 5 measurements.

Gut looping

Dissections were made of 10 individuals from each of the sample sites and the patterns of the loops of the mid and hindgut were examined following Ridgway (1994). Quantification of the gut looping involved describing the direction of the coiling of the top X loop, the relative length of the Y loop, and the length of the Z loop. The structure of the gut and the position of the various loops are shown in Fig. 4. Four measurements (Fig. 4) were taken from the gut loops of each individual and 3 ratios calculated: (a) Z2/Z1 = ratio indicating the relative length of the Z loop, (b) LenX/Z1 = ratio indicating the length of X loop relative to the length of the visceral mass, and (c) WidX/LenX = ratio of the width of the X loop relative to its length. The measurements, even after transformation, did not satisfy the assumptions of ANOVA, therefore to test for any significant differences between the populations, the non-parametric Kruskal–Wallis test and the Wilcoxon 2-sample test were performed based on the methods outlined by Dunn (1964).

Sperm microstructure

Due to logistical constraints, sperm could be examined only from Groen River, Kommetjie, Kalk Bay, Port Elizabeth, Green Point and Mapelane, thus covering 2 sites from each of the 3 coastal regions. Small portions of the testis of 5 males per site were placed in 4% formalin and other portions in 2.5% gluteraldehyde in filtered sea water (except at Mapelane where samples were fixed in 2.5% gluteraldehyde only).

The samples fixed in 4% formalin were prepared for light microscopy. Ten replicate measurements were made of the sperm head and mid-piece length for 5

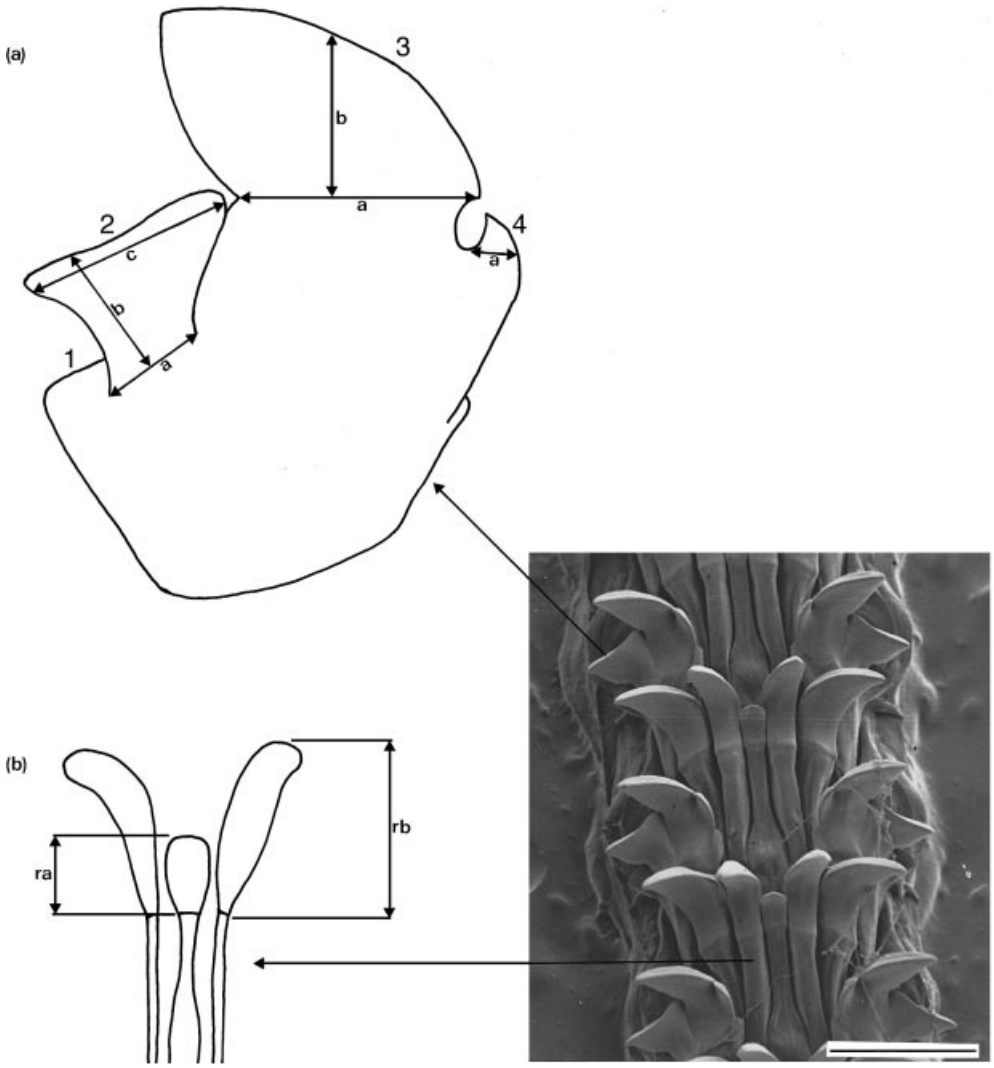

Fig. 3. Scanning electron micrograph of the radular morphology of *Patella granularis* with diagrammatic representations of (a) the pluricuspid tooth, and (b) the rachidian and first lateral teeth, showing the dimensions measured. See Methods for identities of the measurements. Scale bar: 200 μm.

individuals from each population. All measurements were carried out under oil immersion using a Nikon Filar micrometer eyepiece. Nested analysis of variance was used (Statistica for Windows Release 5.1, StatSoft Inc., 1996) to test for any significant differences between the populations.

The samples fixed in 2.5% gluteraldehyde were washed for 30 min in 0.2 M sodium cacodylate buffer (pH 7.2) and postfixed for 90 min in 1% osmium tetroxide in 0.2 M sodium cacodylate buffer, dehydrated through a graded ethanol series and embedded in a Taab/Araldite resin mixture (Cross, 1989) via propylene oxide. Thin sections were cut and stained in 5% aqueous uranyl acetate for 30 min and lead citrate for 3 min. The sections were examined with a JEOL 1210 transmission electron microscope (TEM) at 80 kV. The following measurements, using the Image Measuring System (IMS) of the JEOL 1210 TEM (absolute accuracy to within 3%), were taken from 10 mid-longitudinal sections per population from the TEM images: (a) length of the nucleus, (b) length of the acrosome and (c) length of the subacrosomal space (Fig. 5). Differences between the populations were tested using ANOVA and Tukey's honestly significant difference test.

Electrophoretic analyses

Genetic variation at 16 allozyme loci was examined using standard horizontal starch gel electrophoresis (see Harris & Hopkinson, 1976) (for sample sizes see Table 4). Foot muscle and mantle tissue were prepared from each individual. Tissue samples were roughly minced using a scalpel and then homogenized in 0.01 M Tris buffer (pH 8.0) using a glass rod attached to a portable, variable speed motor. Prior to electrophoresis the homogenate was centrifuged at 2500 **g** for 5 min. Gels were prepared with 12.5% hydrolysed potato starch. The following buffer systems were used: (a) Tris-citrate-lithium hydroxide-borate buffer (Ridgeway, Sherburne & Lewis, 1970), (b) Tris-borate-EDTA buffer (Markert & Faulhaber, 1965) and (c) Tris-citrate buffer (Whitt,

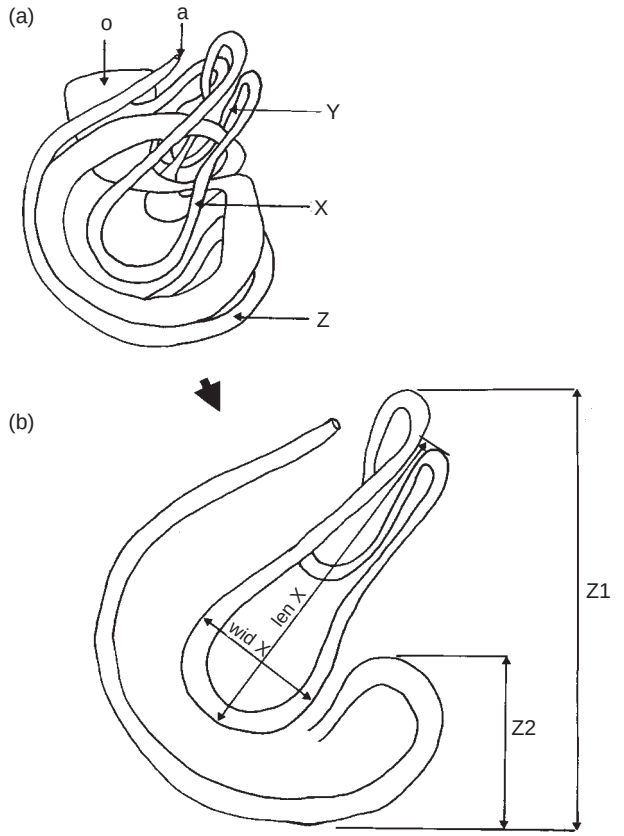

Fig. 4. Diagrammatic representation of the gut loops of *Patella granularis* after complete dissection from the digestive gland showing (a) the relative positions of the X, Y, and Z loops, and (b) the dimensions of the X and Z loops measured. See Methods for identities of the measurements. a, anus; o, oesophagus.

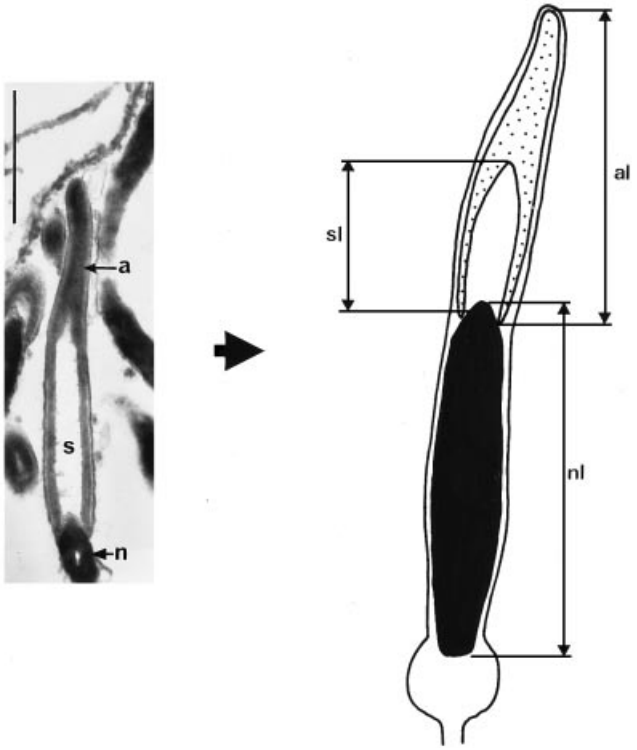

Fig. 5. Mid-longitudinal TEM section through the spermatozoa of *Patella granularis* with a diagrammatic representation of the dimensions measured. a, acrosome; al, length of acrosome; n, nucleus; nl, length of nucleus; s, subacrosomal space; sl, length of subacrosomal space. Scale bar: 1 μm.

Table 1. Enzymes, locus abbreviations, buffer systems and tissue types used (see text for details of buffers)

Enzyme (abbreviation)	E.C. number	Locus	Buffer system	Tissue
Arginine kinase (ARK)	2.7.3.3	ARK-2	(a)	Foot
Glucose-6-phosphate (GPI)	5.3.1.9	GPI-1	(a)	Foot
Phosphoglucomutase (PGM)	2.7.5.1	PGM-1	(a)	Foot
Sorbitol dehydrogenase (SDH)	1.1.1.14	SDH-1	(a)	Foot
Superoxide dismutase (SOD)	1.15.1.1	SOD-1	(b)	Mantle
Malic enzyme (ME)	1.1.1.40	ME-1	(b)	Mantle
		ME-2	(b)	Mantle
Mannose-6-phosphate isomerase (MPI)	5.3.1.8	MPI-1	(b)	Mantle
Peptidase - Glycyl-leucine (GL) as substrate	3.4.-.-	GL-2	(b)	Foot
Peptidase - Leucyl-glycyl-glycine (LGG) as substrate	3.4.-.-	LGG-1	(b)	Foot
Peptidase - Phenylalanine-proline (PHP) as substrate	3.4.-.-	PHP-1	(b)	Foot
Hexokinase (HEX)	2.7.1.1	HEX-1	(b)	Foot
Malate dehydrogenase (MDH)	1.1.1.37	MDH-1	(c)	Mantle
		MDH-2	(c)	Mantle
Isocitrate dehydrogenase (IDH)	1.1.1.42	IDH-2	(c)	Mantle
Aspartate aminotransferase (GOT)	2.6.1.1	GOT-1	(c)	Mantle

Table 2. Shell texture and pedal coloration in the 13 populations of *Patella granularis*

	Swakopmund	Luderitz	Groen River	Kommetjie	Kalk Bay	Port Elizabeth	East London	Dwesa	Coffee Bay A	Coffee Bay B	Oslo Beach	Green Point	Mapelane
Nodule colour	white	white	white	white	white	white	white	white	white	black	black	black	black
No. of costae	-	44.8±0.48	-	44.9±0.75	43.5±0.57	44.2±0.60	44.0±0.56	44.8±0.67	42.9±0.55	42.4±0.41	45.3±0.70	43.1±0.53	42.1±0.52
No. of nodules per costae:													
anterior	-	5.8±0.14	-	6.2±0.18	5.2±0.09	5.8±0.18	5.5±0.15	6.0±0.16	6.5±0.15	6.2±0.17	6.4±0.15	6.4±0.18	6.2±0.17
posterior	-	6.3±0.18	-	5.7±0.18	5.3±0.11	6.4±0.15	6.1±0.16	6.7±0.15	6.9±0.16	6.7±0.21	6.6±0.18	6.7±0.26	6.7±0.19
Foot colour: underside	creamy-orange	creamy-orange	creamy-orange	creamy-orange	creamy-grey	grey	creamy-grey	creamy-grey	grey	grey	creamy-orange	grey	grey
side	grey	creamy-grey	grey	cream	creamy with grey fleck	creamy with grey flecks	creamy with grey flecks	creamy-grey	grey	grey	grey	grey	grey

Table 3. Mean and standard error of the shell dimensions measured from 13 populations of *Patella granularis* (see Methods for explanation of abbreviations)

	Population	SL	SW	SH	WT	VOL
1	Swakopmund	35.83±0.63	27.12±0.60	14.25±0.39	4.06±0.24	3.96±0.23
2	Luderitz	39.32±0.94	29.71±0.78	17.06±0.83	4.91±0.45	5.71±0.48
3	Groen River	28.64±0.47	21.59±0.38	11.48±0.46	1.69±0.09	1.82±0.11
4	Kommetjie	36.48±0.47	29.05±0.40	13.64±0.29	3.29±0.16	3.87±0.17
5	Kalk Bay	30.94±0.37	23.21±0.33	9.02±0.18	1.63±0.07	1.63±0.07
6	Port Elizabeth	23.77±0.27	17.41±0.21	8.09±0.16	0.91±0.03	0.98±0.03
7	East London	26.21±0.37	18.17±0.27	9.13±0.18	1.25±0.05	1.32±0.06
8	Dwesa	25.60±0.65	17.33±0.52	8.66±0.36	1.08±0.10	1.19±0.11
9	Coffee Bay A	21.87±0.92	15.51±0.59	8.04±0.38	0.86±0.10	0.85±0.10
10	Coffee Bay B	16.62±0.40	11.88±0.28	6.32±0.21	0.38±0.03	0.43±0.03
11	Oslo Beach	26.64±0.63	17.90±0.50	9.45±0.30	1.30±0.12	1.17±0.12
12	Green Point	21.39±0.59	15.66±0.47	8.89±0.27	0.89±0.07	0.87±0.06
13	Mapelane	18.34±0.62	13.39±0.48	7.17±0.28	0.42±0.04	0.59±0.06

1970). All gels were run for between 4 and 5 h at a constant current of 30 mA. Gels were cut into 4 slices, which were then stained for different enzymes using specific histochemical stains (Shaw & Prasad, 1970; Harris & Hopkinson, 1976). The enzymes assayed, the buffer systems, and the tissue used are listed in Table 1.

The electrophoretic data were analysed using the programme BIOSYS-1 Release 1.7 (Swofford & Selander, 1981). Allele and genotype frequencies were calculated. Deviations of genotype frequencies from Hardy–Weinberg equilibrium were measured using the Levene (1949) test which makes allowance for small sample sizes. The average unbiased genetic identity (I) and distance (D) among the populations were calculated from the allele frequencies according to Nei (1978), and these were used to construct a dendrogram using the unweighted pair-group method with arithmetic mean (UPGMA) clustering algorithm (Sneath & Sokal, 1973).

RESULTS

Morphological analyses

Shell texture and pedal coloration

On the whole the colour of the nodules showed a clear difference between the populations east versus those west of Coffee Bay, with shells from the west and south coasts having nodules of a lighter (off-white) pigmentation than the background coloration of the shell, and shells from the east coast having nodules of a darker (black) pigmentation than the background coloration of the shell (Table 2). The shells from Coffee Bay were variable, with some having white nodules, and others black nodules and they were therefore treated as separate populations, Coffee Bay A and Coffee Bay B.

There was very little variation in the number of costae between the 13 populations, with the only significant differences being between Mapelane and both Oslo Beach and Kommetjie (ANOVA, $P<0.05$). The number of the nodules on the anterior costae did show differences, with shells from Kommetjie, Dwesa, Coffee Bay A, Coffee Bay B, Oslo Beach, Green Point and Mapelane differing significantly from Kalk Bay (ANOVA, $P<0.05$). East London also differed significantly from Coffee Bay A, Oslo Beach and Green Point. The number of nodules on the posterior costae also showed differences with Luderitz, Port Elizabeth, Dwesa, Coffee Bay A, Coffee Bay B, Oslo Beach, Green Point and Mapelane differing significantly from Kalk Bay, and Dwesa, Coffee Bay A, Coffee Bay B, Oslo Beach, Green Point and Mapelane differing significantly from Kommetjie (ANOVA, $P<0.05$).

The pigmentation of the underside and the side of the foot was very variable but the east coast populations did tend to have a darker pigmentation than the west coast populations, with the exception of Oslo Beach, which had creamy-orange undersides of the foot. Thus, the number of costae, the number of nodules per costae and the pigmentation of the underside and side of the foot did not show any clear trend from the west to east coasts, and therefore were not considered to be useful definitive characters. However, the colour of the nodules does appear to be a useful distinguishing character.

Shell morphometrics

Examination of shell lengths (Fig. 6) shows that size tends to decline from west to east (Table 3). Figure 7 shows a plot of the first two canonical variables for the pooled populations from the three regions. It is clear that there is major overlap between the groups, as indicated by the overlap of the convex hulls, with the first two canonical variables only accounting for 79.9% of the variance used to distinguish between the populations. However, despite the overlap, some differentiation is evident in that the trend for the first canonical variable reflects the fact that the west coast shells are larger than the east coast shells. To evaluate population differences, the proportion of correctly identified specimens in each population was calculated. The classification function of the 16 populations indicated that, based on SL, SW, SH, WT and VOL, only 57.4% of the individuals were correctly identified. Of the 228 west coast specimens examined, 83.8% were correctly reassigned to the west coast group, with 13.6% being assigned to the south coast populations and only six individuals (2.6%) being placed into the east coast populations. When dealing with the south coast populations, 149 (75.6%) of the 197 specimens were correctly reassigned to this group, 10.7% were assigned to the west coast populations and 13.7% to the east coast populations. Only 64.8% of the east coast populations were reassigned correctly, with 21.6% being placed in the south coast populations and 13.6% in the west coast populations. Thus, it appears that with the exception of shell length, no obvious characteristic of the shell mor-

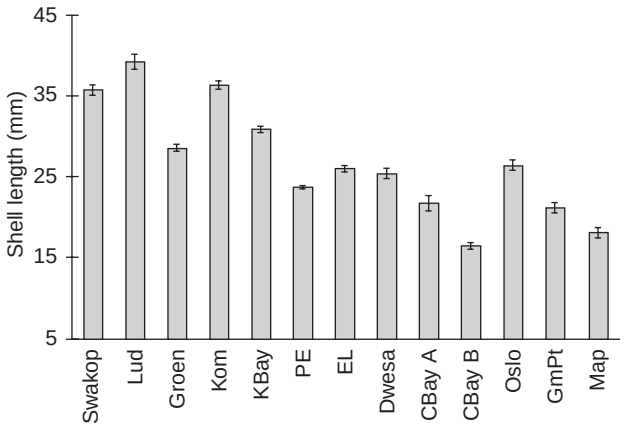

Fig. 6. Mean (± SE) of the lengths of the shells of *Patella granularis* from the 13 populations along the southern African coast. (Populations are arranged in geographical order around the coast from west to east.)

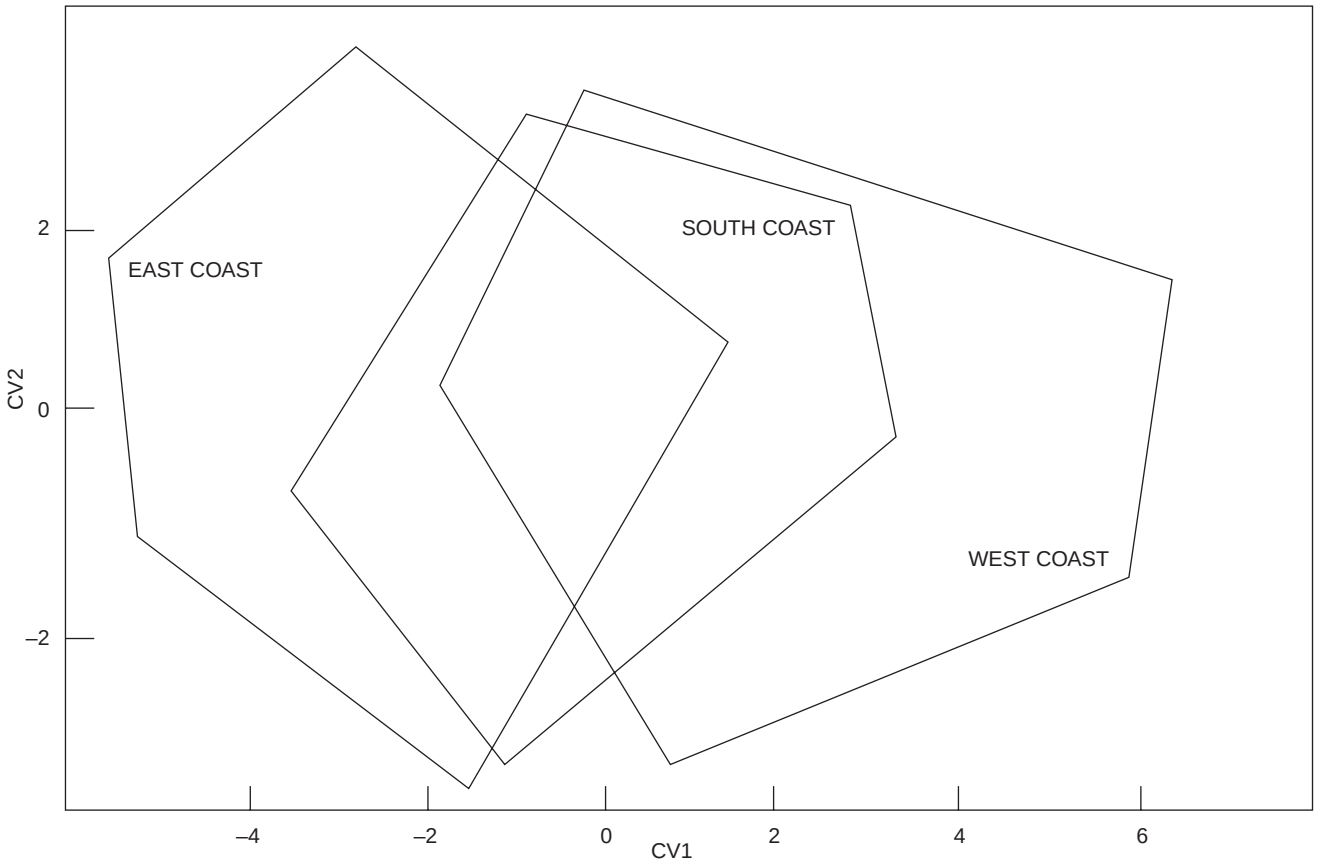

Fig. 7. Plot of the first two canonical variables for the west, south, and east coast populations of *Patella granularis*. The outlines are convex hulls surrounding the three coastal regions.

Table 4. Mean and ranges of the five measurements taken from the radula of five populations of *Patella granularis*

	Groen River	Kommetjie	Kalk Bay	Port Elizabeth	Green Point
ra/rb					
mean	0.458	0.486	0.419	0.455	0.388
range	0.437–0.485	0.409–0.549	–	0.429–0.481	0.353–0.411
C4a/C3a					
mean	0.342	0.262	0.363	0.339	0.426
range	0.308–0.383	0.215–0.325	0.321–0.397	0.322–0.355	0.383–0.472
C2a/C3a					
mean	0.680	0.620	0.582	0.696	0.786
range	0.612–0.715	0.594–0.645	0.437–0.720	0.646–0.746	0.722–0.823
C2a/C2c					
mean	0.541	0.513	0.646	0.663	0.816
range	0.393–0.611	0.425–0.707	0.558–0.796	0.631–0.695	0.637–0.974
C2b/C3b					
mean	1.093	1.009	0.664	0.806	0.701
range	0.933–1.186	0.887–1.468	0.527–0.761	0.637–0.974	0.674–0.748

phometrics differentiates between the populations from the west, south and east coasts.

Radular morphology

No difference in radula structure was found between the populations from the west (Groen River and Kommetjie), south (Kalk Bay and Port Elizabeth) and east (Green Point) coasts. All five populations had typical docoglossate radulae that could be described by the formula 3 + 1 + (2 + 1 + 2) + 1 + 3 (Powell, 1973). All radulae had three pairs of unmineralized marginal teeth, three pairs of lateral teeth and a single, central rachidian tooth. The lateral teeth can be divided into a pair of large pluricuspid teeth and two pairs of smaller

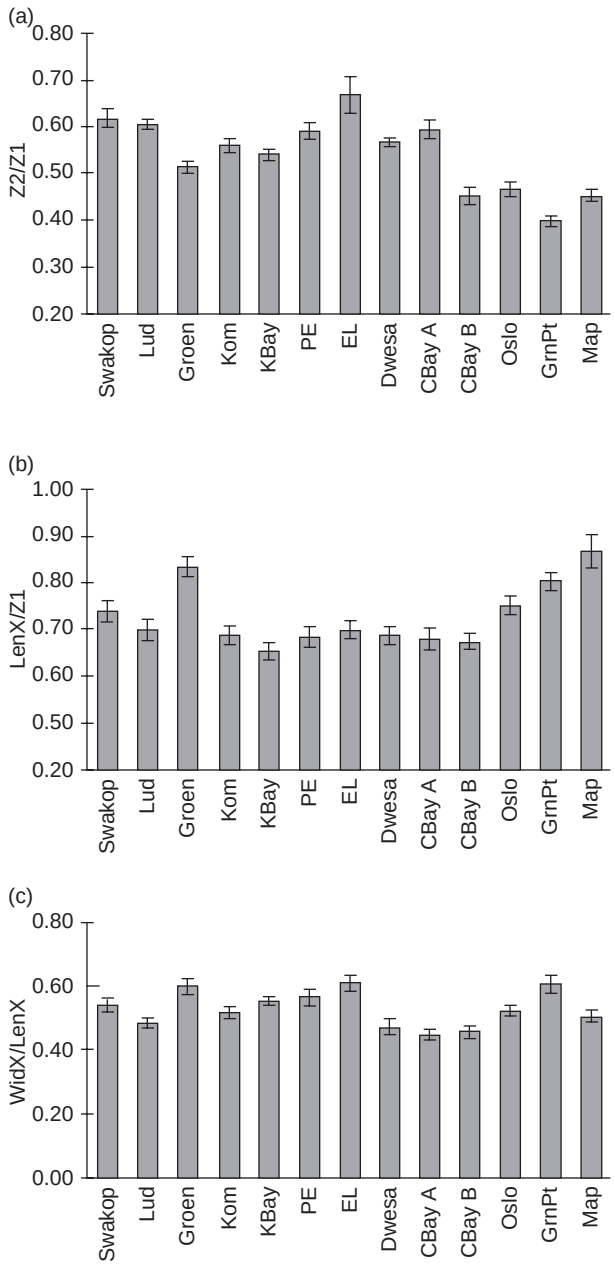

Fig. 8. Gut loop measurements (mean ± SE) for the 13 populations of *Patella granularis*. See Methods for abbreviations.

unicuspid teeth. The pluricuspid tooth has four cusps, with cusp 3 being pointed and larger than the other cusps. Table 4 shows the mean and the lower and upper ranges of the five measurements taken from the radula. Kruskal–Wallis and Wilcoxon two-sample tests revealed no significant differences between the west, south and east coasts. However, despite the fact that the mean values for ra/rb, C4a/C3a, C2a/C3a and C2a/C2c for Green Point differ considerably from the other populations, there is overlap in the ranges of the populations. The small samples sizes make comparison between the populations very difficult but the results do suggest that the Green Point population differs from the other populations.

Gut looping

In all 13 populations the X loop of the gut was coiled neither clockwise nor anticlockwise (Ridgway, 1994). The Y loop was less than 30% of the length of the X loop, and was therefore classified as being 'relatively short' in all populations. The mean and standard error of the ratios measured from the X and Z loops for each of the 13 populations are shown in Fig. 8. The length of the Z loop (Fig. 8a) shows that individuals from Coffee Bay B, Oslo Beach, Green Point and Mapelane had relatively shorter Z loops than the other nine populations. Swakopmund, Luderitz, Port Elizabeth, East London, Dwesa and Coffee Bay A differed significantly (Kruskal–Wallis, $P<0.05$) from Coffee Bay B, Oslo Beach, Green Point and Mapelane for this character. Kommetjie and Kalk Bay differed significantly (Kruskal–Wallis, $P<0.05$) from only Green Point. The length of the X loop relative to the visceral mass (Fig. 8b) showed some significant differences (Kruskal–Wallis, $P<0.05$), with specimens from Groen River and Mapelane having longer than normal X loops. Individuals from Groen River differed significantly from those sampled at Kommetjie, Kalk Bay, Port Elizabeth, Dwesa, Coffee Bay A and Coffee Bay B. Mapelane differed significantly (Kruskal–Wallis, $P<0.05$) from Luderitz, Kalk Bay, Port Elizabeth, Dwesa, Coffee Bay A and Coffee Bay B. The width relative to the length of the X loop (Fig. 8c) was almost constant throughout all the populations with only Luderitz, Dwesa, Coffee Bay A and Coffee Bay B differing significantly (Kruskal–Wallis, $P<0.05$) from East London and Green Point. Thus, despite some significant differences, the patterns and loops of the mid and hindgut generally remain fairly constant throughout the populations, with the exception of the relatively shorter Z loop in the four northernmost east coast populations.

Sperm microstructure

Sperm from all of the six populations examined were of similar shape, all having heads that were elongated and therefore classified as Type II sperm (Hodgson, Ridgway *et al*., 1996). Figure 9a shows the mean and the standard error of the sperm head and mid-piece length (μm) for individuals from five of the populations. Individuals from Kommetjie had significantly longer sperm heads (ANOVA, $P<0.05$) than those from the Groen River, Kalk Bay, Port Elizabeth and Green Point populations, and individuals from Groen River and Green Point were significantly longer (ANOVA, $P<0.05$) than the Kalk Bay population, but there was no clear-cut pattern distinguishing the different coastal regions. Figure 9b shows the sperm nucleus length (μm) for the six populations examined. No significant differences were detected between any of the populations (ANOVA, $P>0.05$). Figure 9c and 9d show the acrosomal length and the length of the subacrosomal space, respectively. In both cases the populations at Green Point and Mapelane exhibited significant differences

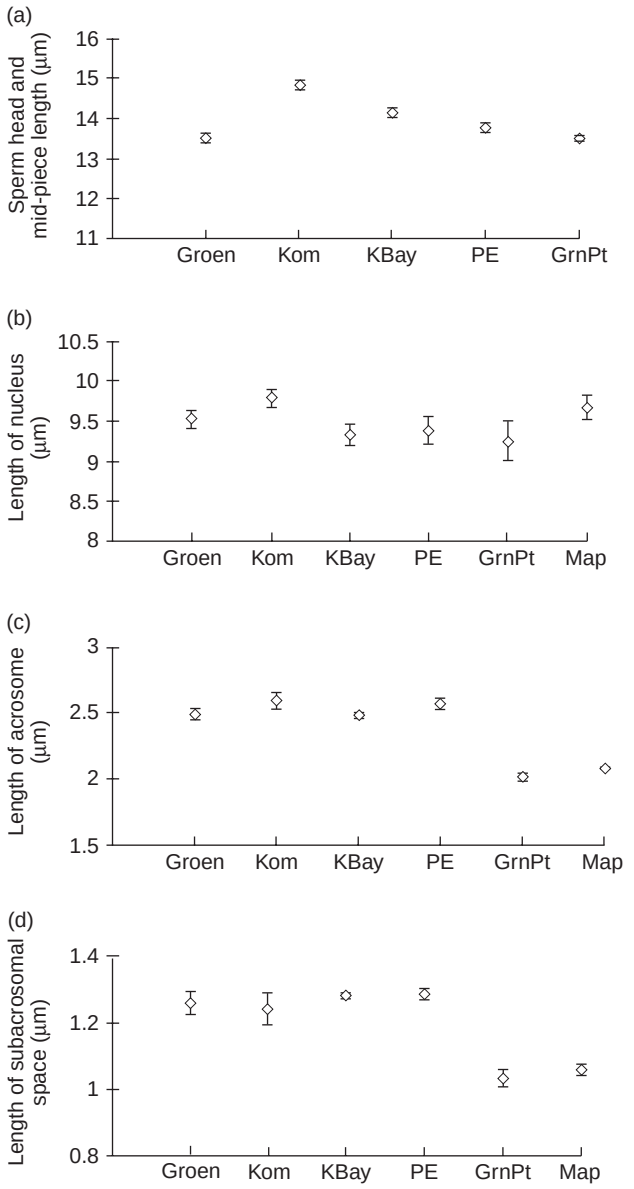

Fig. 9. Sperm microstructure measurements (mean ± SE) for the six populations of *Patella granularis* examined.

from those at Groen River, Kommetjie, Kalk Bay and Port Elizabeth (ANOVA, $P < 0.05$). Thus, the length of the sperm head and mid-piece length and the length of the nucleus do not appear to be useful definitive characters, whereas the acrosomal and subacrosomal space length do seem to be useful characters.

Electrophoretic analyses

The allele frequencies for the polymorphic loci and those showing fixed allele differences detected in the 13 populations of *Patella granularis* are listed in Table 5. Six of the loci (SDH-1, SOD-1, ME-1, MPI-1, HEX-1, MDH-2) were consistently monomorphic for all of the populations and have therefore been omitted from Table 5. Ten loci were polymorphic or showed fixed allele differences, with the number of alleles per locus ranging from two in ARK-2, ME-2, GL-2, PHP-1, and MDH-1 to four in GPI-1. Within any one population, the maximum number of alleles per locus was four (for GPI in Kommetjie and East London specimens).

Tests for Hardy–Weinberg equilibrium (using the Levene (1949) correction for small sample size) indicated that of the 36 cases of polymorphism (using the 0.99 criterion) encountered at all loci and all populations, nine (25%) exhibited significant deviations (χ^2, $P < 0.05$). The deviations occurred at four loci, and were all due to a significant deficiency in the number of heterozygotes. The genotype frequencies that were not in Hardy–Weinberg equilibrium were for GPI-1 at Port Elizabeth, PGM-1 at East London, ME-2 at East London, Coffee Bay A, Coffee Bay B, Oslo Beach, Green Point and Mapelane, and IDH-2 at Dwesa.

ARK-2, GL-2, PHP-1, and MDH-1 were fixed for one particular allele in the populations at Coffee Bay B, Oslo Beach, Green Point and Mapelane, and for another allele in the remaining populations. Using Avise's (1975) definition of a diagnostic locus, these four loci can be considered to be taxonomically diagnostic.

Cluster analysis (Fig. 10) of Nei's (1978) unbiased genetic identities (I) between all pairs of populations (Table VI) revealed two distinct groups. 'Group A' consists of nine populations (Swakopmund, Luderitz, Groen River, Kommetjie, Kalk Bay, Port Elizabeth, East London, Dwesa and Coffee Bay A), with the mean identity within this group being 0.994. 'Group B' consists of four populations (Coffee Bay B, Oslo Beach, Green Point and Mapelane) which are genetically identical (mean identity of 1.000). 'Group A' separated from 'group B' at an I value of 0.528 (Nei's (1978) genetic distance (D) = 0.638). Thus, at Coffee Bay both genetic forms were found to occur sympatrically.

DISCUSSION

The isozyme and morphological variations in the 13 populations of *Patella granularis* along the coast of southern Africa show that the west coast populations do not form a distinct group as originally hypothesized. This finding does not coincide with the shell structural differences reported by Cohen (1988) and Cohen & Branch (1992), who examined populations of *P. granularis* from Port Nolloth on the west coast through to Durban on the east coast. They showed that the west coast populations differ substantially in shell structure from the south and east coast populations. In particular, the unique presence of a calcitic $m - 2$ layer distinguishes all west coast populations from those elsewhere. However, there is no evidence at all to suggest that west coast populations are genetically separable from those of the south coast (Fig. 10). Rather, the most striking result obtained in this study is the clear genetic distinction between, on the one hand, populations of *P. granularis* from the west, south, and southern east coasts (Swakopmund, Luderitz, Groen River, Kom-

Table 5. Distribution of allele frequencies at 10 loci in 13 populations of *Patella granularis*. *N* = sample size

Locus	Swakopmund	Luderitz	Groen River	Kommetjie	Kalk Bay	Port Elizabeth	East London	Dwesa	Coffee Bay A	Coffee Bay B	Oslo Beach	Green Point	Mapelane
ARK2													
N	15	20	15	25	11	15	15	15	18	22	20	25	20
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	-	-
B	-	-	-	-	-	-	-	-	-	1.000	1.000	1.000	1.000
GPI-1													
N	15	20	30	35	26	30	30	15	18	22	20	35	20
A	1.000	0.925	1.000	0.900	0.904	0.917	0.800	0.933	1.000	-	-	0.029	-
B	-	-	-	0.043	0.038	0.083	0.083	0.033	-	-	-	-	-
C	-	-	-	0.014	-	-	0.017	-	-	-	-	-	-
D	-	0.75	-	0.043	0.058	-	0.100	0.033	-	1.000	1.000	0.971	1.000
PGM-1													
N	15	20	10	17	10	10	10	15	18	22	20	20	20
A	0.967	1.000	0.950	0.765	0.800	1.000	0.850	0.967	0.944	-	-	-	-
B	0.033	-	0.050	0.235	0.200	-	-	0.033	0.056	-	-	-	-
C	-	-	-	-	-	-	0.150	-	-	1.000	1.000	1.000	1.000
ME-2													
N	15	20	10	25	15	15	15	15	18	17	20	25	15
A	1.000	1.000	1.000	1.000	1.000	1.000	0.867	1.000	0.500	0.118	0.150	0.160	0.250
B	-	-	-	-	-	-	0.133	-	0.500	0.882	0.850	0.840	0.750
GL-2													
N	15	20	10	23	10	15	15	15	18	22	20	23	20
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	-	-
B	-	-	-	-	-	-	-	-	-	1.000	1.000	1.000	1.000
LGG-1													
N	15	20	10	25	15	15	15	15	18	22	20	25	20
A	1.000	0.975	1.000	0.980	1.000	1.000	0.967	1.000	0.972	0.045	-	0.020	0.050
B	-	0.025	-	0.020	-	-	-	-	0.028	0.955	1.000	0.980	0.950
C	-	-	-	-	-	-	0.033	-	-	-	-	-	-
PHP-1													
N	15	20	10	25	10	15	15	15	18	22	20	25	20
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	-	-
B	-	-	-	-	-	-	-	-	-	1.000	1.000	1.000	1.000
MDH-1													
N	15	20	15	25	10	15	15	15	18	22	20	25	20
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	-	-
B	-	-	-	-	-	-	-	-	-	1.000	1.000	1.000	1.000
IDH-2													
N	15	20	10	25	10	10	15	14	18	22	20	25	20
A	0.900	0.900	1.000	0.960	1.000	1.000	1.000	0.643	0.944	0.977	1.000	1.000	1.000
B	0.067	0.100	-	0.040	-	-	-	0.321	0.056	0.023	-	-	-
C	0.033	-	-	-	-	-	-	0.036	-	-	-	-	-
GOT-1													
N	15	20	10	25	10	10	15	15	18	22	20	25	20
A	0.933	1.000	1.000	1.000	0.950	1.000	0.967	1.000	1.000	1.000	1.000	1.000	1.000
B	0.033	-	-	-	-	-	0.033	-	-	-	-	-	-
C	0.033	-	-	-	0.050	-	-	-	-	-	-	-	-

Table 6. Matrix of Nei's (1978) genetic identity (above diagonal) and genetic distance (below diagonal) averaged over 16 loci in 13 populations of *Patella granularis*

	Population	1	2	3	4	5	6	7	8	9	10	11	12	13
1	Swakopmund	*****	1.000	1.000	0.997	0.998	0.999	0.997	0.997	0.985	0.510	0.509	0.515	0.523
2	Luderitz	0.000	*****	0.999	0.997	0.998	0.999	0.997	0.997	0.984	0.521	0.520	0.525	0.533
3	Groen River	0.000	0.001	*****	0.998	0.999	1.000	0.997	0.993	0.985	0.516	0.515	0.521	0.528
4	Kommetjie	0.003	0.003	0.002	*****	1.000	0.997	0.997	0.993	0.982	0.527	0.526	0.532	0.540
5	Kalk Bay	0.002	0.002	0.001	0.000	*****	0.998	0.998	0.992	0.983	0.525	0.524	0.530	0.538
6	Port Elizabeth	0.001	0.001	0.000	0.003	0.002	*****	0.998	0.993	0.984	0.517	0.516	0.521	0.529
7	East London	0.003	0.003	0.003	0.003	0.002	0.002	*****	0.991	0.990	0.552	0.550	0.555	0.562
8	Dwesa	0.003	0.003	0.007	0.007	0.008	0.007	0.009	*****	0.979	0.506	0.504	0.510	0.518
9	Coffee Bay A	0.016	0.016	0.015	0.018	0.017	0.016	0.011	0.021	*****	0.550	0.548	0.552	0.554
10	Coffee Bay B	0.673	0.653	0.662	0.640	0.644	0.660	0.595	0.681	0.597	*****	1.000	1.000	1.000
11	Oslo Beach	0.675	0.654	0.663	0.642	0.646	0.661	0.597	0.685	0.602	0.000	*****	1.000	1.000
12	Green Point	0.664	0.644	0.653	0.632	0.636	0.651	0.588	0.674	0.593	0.000	0.000	*****	1.000
13	Mapelane	0.649	0.629	0.638	0.617	0.620	0.636	0.576	0.674	0.590	0.000	0.000	0.000	*****

metjie, Kalk Bay, Port Elizabeth, East London, Dwesa and Coffee Bay A) and, on the other hand, those from the northern east coast (Coffee Bay B, Oslo Beach, Green Point and Mapelane) (Fig. 10).

The genetic identity value (Nei, 1978) at which 'group A' and 'group B' separated (Fig. 10) was 0.528 which, according to Thorpe (1982), is typical of different but congeneric species. Thorpe (1982) proposed that the critical level for genetic identity values distinguishing between species and genera is about 0.35, with values between congeneric species falling between 0.35 and 0.85. However, even though Thorpe's (1982) proposed separation of species has been widely used and accepted in taxonomic studies, it is only a guide, and should be used with caution. Côrte-Real *et al.* (1996*a*) examined 15 loci and calculated genetic identities of 0.576 between *Patella candei* and *P. caerulea*/*P. depressa*, and 0.642 between *P. caerulea* and *P. depressa*. Based on 19 loci, Cretella *et al.* (1994) calculated genetic identities of 0.635 between *Patella caerulea* and *P. ferruginea*, and 0.641 between *P. ulyssiponensis* (= *P. aspera*) and *P. ferruginea*. Thus, it appears that the genetic identity value of 0.528 obtained in our study between the two distinct groups is in the range obtained between other clearly distinguishable species of patellid limpets, therefore suggesting that two species are involved.

Of greater significance are the fixed allele differences at ARK-2, GL-2, PHP-1 and MDH-1 between 'group A' and 'group B' because there have been a number of other studies on gastropods in which the presence of diagnostic loci has indicated the presence of two co-existing species (Chambers, 1978; Murphy, 1978; Heller & Dempster, 1991).

The most convincing evidence obtained in this study is the co-occurrence of the two genetically distinct forms at Coffee Bay (Table 5). The failure to find a single individual from either genetic grouping possessing the allele of the other at the diagnostic loci indicates no gene flow between the two forms, therefore implying reproductive isolation. In their review on species concepts, McKitrick & Zink (1988) state that Mayr's (1963) 'biological species concept' is frequently faced with difficulties when dealing with allopatric populations. The difficulties arise because it is usually impossible to gauge whether or not, given the opportunity, individuals from allopatric populations would interbreed. The presence of the four diagnostic alleles in this study suggests that 'group A' and 'group B' are genetically isolated, since there is no gene flow between them. Bock (1986) has proposed a different definition for the 'biological species concept', in which he emphasizes the genetic isolation of species and suggests that rather than using reproductive isolation, the lack of gene flow between the groups should be used to define the bounds of a species. Thus, the presence of the fixed allele differences between 'group A' and 'group B' and the co-existence of the two genetic forms at Coffee Bay (Table 4) provides clear evidence that there is no gene flow between the two groupings, and clearly indicates that two species are involved.

The lack of gene flow between the south and southern east coast populations and the northern east coast populations in this study might possibly be attributed to the marked discontinuity of the inshore water characteristics in the Mbashe area just north of Dwesa (Beckley & van Ballegooyen, 1992). The upwelling of cool Indian Ocean central water onto the shelf in the Mbashe area has been suggested to control the pelagic environment on the east coast (Beckley & Hewiston, 1994). *P. granularis* is a broadcast spawner, and upwelling off Mbashe may have provided a sufficient barrier to the flow of gametes and larvae between the southern and northern east coast populations to have allowed speciation to take place. However, on the other hand, there are much stronger upwelling cells on the west coast, yet these have failed to lead to genetic isolation between any of the west and south coast populations. The strongest upwelling cell on the entire coastline is at Luderitz (Shannon, 1985), yet the populations of *P. granularis* at Swakopmund and Groen River are genetically virtually indistinguishable.

The most striking distinguishing feature obtained from the morphological analyses was the colour of the nodules on the costae of the shells. The shells from the

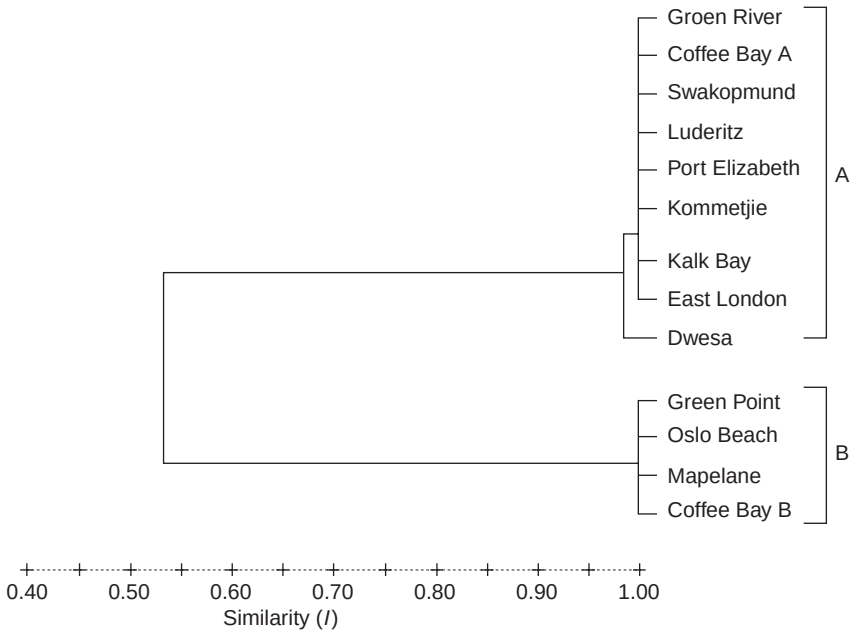

Fig. 10. UPGMA dendrogram derived from Nei's (1978) genetic identity based on 16 loci for 13 populations of *Patella granularis*.

genetic 'group A' have nodules which are whitish in colour whereas the shells from 'group B' have nodules which are black in colour (Table 2). The colour of the nodules therefore provides a consistently useful diagnostic morphological character with which to distinguish the two genetic groupings using external appearances.

Despite the clear genetic differentiation and the difference in nodule coloration, few other morphological distinctions can be drawn between 'group A' and 'group B'. Although the use of soft part morphology has been found to be a useful discriminatory tool in other studies (Cretella *et al*., 1994), in this study the colour of the underside and the side of the foot showed no diagnostic features (Table 2). The comparison of the radulae also revealed no differences, with all the populations examined having the same overall radular structure and relative dimensions (Table 4). All the populations examined occupied the same mid- to high-shore zone on rocky shores and, being generalist intertidal grazers (Branch, 1981), all populations probably use their radular apparatus in similar ways (Hawkins *et al*., 1989).

The pattern of the loops of the mid and hindgut has been used to distinguish between families and genera of patellogastropods (Lindberg, 1988) but it has not been extensively used at the species level (Ridgway, 1994). The patterns of the gut loops of the individuals from the sample populations showed little variation (Fig. 8), with only the length of the Z loop being useful to distinguish between the two genetic groupings, being significantly shorter in 'group B' than in 'group A'.

Spermatological studies have also proven to be a useful technique in archaeogastropod and patellogastropod taxonomy (Healy, 1988; Hodgson & Bernard, 1989; Hodgson & Chia, 1993; Hodgson *et al*., 1996) and proved to be highly informative in this study. Although the sperm head and mid-piece length and the length of the nucleus showed no differences, the lengths of the acrosome and subacrosomal space (Fig. 9) did reveal 'group B' has significantly smaller acrosomes and subacrosomal spaces than 'group A'.

The lengths of the shells around the coast coincide with the findings of Stephenson (1937), with the west coast individuals attaining a larger size than those from the south and east coasts (Fig. 6). A strong intertidal primary productivity gradient on rocky shores exists around the coast of South Africa (Bustamante *et al*., 1995), with the production being highest on the west coast and declining along the south and east coasts. This distinctive west to east productivity gradient is closely related to the oceanographic conditions off the South African coast, particularly the incidence of upwelling, and is correlated with the maximum size attained by *P. granularis* (Bustamante *et al*., 1995). The largest *P. granularis* are recorded on offshore islands, where the intertidal algal productivity is further enhanced by the runoff of guano from bird colonies (Bosman & Hockey, 1988; Branch *et al*., 1987). Furthermore, the size of *P. granularis* is influenced by the density of barnacles on the shore (Branch, 1976). As body size is related to environmental factors it is very unlikely that size has any taxonomic value in distinguishing populations of *P. granularis*.

The discriminant functions analysis on the shell morphometric data revealed no distinctive groupings (Fig. 7), with only a 57.4% chance of correctly reassigning individuals to a particular population. The overlap can be attributed to the fact that the shape of limpet shells can be modified by a wide variety of

environmental influences. The most commonly reported factors influencing shell shape include exposure to wave action (Bacci & Sella, 1970; Simpson, 1985), zonation on the shore (Branch, 1981) and desiccation (Lowell, 1984). The number of costae on the shell and the number of nodules per costa did show differences between populations, but not in a manner that coincided with the groupings of the genetic data (Table 2). Thus, the variation in shell morphometrics between the populations in this study can probably be attributed to differing environmental influences between the sample sites.

Thus, the results from this study reveal that, despite striking morphological similarities between populations of the limpet currently lumped under the name '*Patella granularis*', the four northernmost populations on the east coast (Coffee Bay B, Oslo Beach, Green Point and Mapelane) represent a gene pool that is distinct from the west (Swakopmund, Luderitz, Groen River and Kommetjie), south (Kalk Bay, Port Elizabeth and East London), and southern east coast (Dwesa and Coffee Bay A) populations, therefore indicating that two reproductively isolated species are involved. Krauss (1848) described a species of limpet from Natal, on the east coast of South Africa, which he named *Patella natalensis*. He based his classification solely on shell morphological characteristics, concluding that it is closely related to *P. granularis* but that it is smaller and has black nodules on the shell. Krauss (1848) also described another species with black nodules on the shell which he called *Patella echinulata*. However, *P. echinulata* was recorded as being found in Table Bay, which is on the west coast of the Cape Peninsula of South Africa. Bartsch (1915), in his account on molluscan taxonomy, which was based solely on shell morphology, recognized both *P. natalensis* and *P. echinulata*. Turton's (1932) account also recognized *P. natalensis* but he placed *P. echinulata* as a subspecies of *P. natalensis*. However, Tomlin & Stephenson (1942), after examining only the shell morphology of the type specimens of *P. natalensis* and *P. echinulata*, classified both as small specimens of *P. granularis*. Thus, Tomlin & Stephenson (1942) did not consider the different colour of the nodules to be sufficient to warrant the recognition of separate species. In addition, in the more recent reviews on patellid taxonomy, Koch (1949), Christiaens (1973) and Powell (1973) report that only one species, *P. granularis*, is present.

The present study conclusively shows that the populations of '*P. granularis*' from the four northernmost sites on the east coast of South Africa are not in fact this species, but rather a cryptic and closely related sibling species. These east coast populations provide evidence for Krauss's (1848) classification of *P. natalensis* as a distinct species distinguishable from *P. granularis* in KwaZulu-Natal and northern Transkei. *P. natalensis* differs from *P. granularis*, in its new restricted form, in that the nodules on the costae of the shell are black, not white; the Z loop of the gut is smaller; and the length of the acrosome and the subacrosomal space of the sperm is shorter. Thus, it is evident that two species, *P. granularis* and *P. natalensis*, are present, the definition and resolution of which will be described in a separate paper.

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