A genetic and morphological analysis of species complexes within the genus *Patella*

by

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Thesis submitted for the degree of Master of Science

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University of Cape Town

July 1998

"It is my hope that an increased awareness of the sea, which for thousands of years has taught man wisdom, will inspire him once more those thoughts and actions that will preserve the balance of nature and maintain life itself."

Captain Jacques Cousteau

To my mother, Gillian Ridgway

Declaration

This thesis documents original research, carried out in the Marine Biology Research Institute (University of Cape Town) and the South African Museum. The work has not been submitted in whole or in part for a degree at any other university. All assistance that I have received has been fully acknowledged.

Tyrone M. Ridgway

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Abstract

Ridgway, T.M. 1998. A genetic and morphological analysis of species complexes within the genus *Patella*. Unpublished M.Sc. thesis, University of Cape Town, South Africa.

Limpets of the genus *Patella* are very common intertidal molluscs that have a widespread world-wide distribution. Despite this, the systematics of the genus is still in a state of confusion. However, in comparison to other regions, the southern African representatives of *Patella* are fairly well characterised. Nevertheless, some long-standing taxonomic uncertainties still exist for certain of the southern African species. This thesis combines both morphological and genetic techniques to solve these uncertainties.

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 of no diagnostic use in distinguishing between populations. Discriminant functions analysis based on shell morphometrics only partially discriminate the 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. These northern-most populations are also distinguished by having significantly shorter Z looping of the gut than in the other populations. No other differences in soft-part morphology, or in radular structure were detected between the populations. 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 of 0.528 as well as four diagnostic alleles between the four northern-most populations from the east coast compared with those to the south and west. The two genetically distinct forms occur sympatrically at Coffee Bay on the east coast. It was concluded the two groupings are 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*. These east-coast specimens correspond to Krauss's description of *P. natalensis*, and this species is thus re-described.

Patterns of genetic and morphological variation among eight populations of *Patella barbara* along the coast of South Africa were examined to see whether reported differences in shell morphology and behaviour between the west and east coasts are mirrored by other morphological and/or genetic differences. Morphological analyses of radular structure, gut-loop coiling and sperm microstructure revealed no differences between the populations. Discriminant functions analysis based on shell morphometrics showed major overlap between populations and failed to separate them. Electrophoretic analysis of 17 enzyme loci gave an overall genetic identity value of 0.995, a value which lies within the generally accepted range for conspecific populations. In two of the populations, at Dwesa and Arniston, individuals were found to be 'gardening' algal resources. At Clovelly, a 'normal' form of *P. barbara* was found on the rocks, whereas a reduced and

tall-shelled form occurred on the ascidian *Pyura stolonifera*, demonstrating phenotypic morphological plasticity. It was concluded that different populations of *P. barbara* are both morphologically indistinguishable and genetically homogenous along the coast of South Africa, thus providing good evidence for widespread gene flow.

Finally, the species boundaries between *Patella* cf.. *miniata* (sometimes called *P. adansonii*), *P. miniata miniata*, *P. miniata sanguinans*, and *P. compressa* were examined to resolve the taxonomic uncertainty surrounding the '*Patella miniata*' species complex in southern Africa. In addition, a population of *P. safiana* from southern Angola was included as a reference population. An electrophoretic analysis of 16 enzyme loci failed to distinguish *P.* cf. *miniata* from *P. miniata miniata*, so that they are regarded as conspecifics. *Patella adansonii* (originally described from and known only from shell material) is indistinguishable from the group I initially called *P.* cf. *miniata*, so I regard it as synonymous with *P. miniata*. The electrophoretic analysis did reveal four distinctive groupings. The *P. miniata miniata* populations separated from the *P. compressa* populations at $I = 0.85$; the *P. miniata sanguinans* populations clustered out at $I = 0.79$; and *P. safiana* separated at $I = 0.69$. Phylogenetic analyses, using *P. safiana* as an outgroup, showed that *P. miniata miniata*, *P. miniata sanguinans* and *P. compressa* form a closely related monophyletic group in which *P. miniata miniata* and *P. compressa* are more closely related to each other than either one of them is to *P. miniata sanguinans*. Morphological analyses revealed differences that mirrored the differences detected by the allozymes. All these species had radulae with identical formulae. However, the cusps of *P. compressa* have concave cutting edges, whereas those of *P. miniata miniata* and *P. miniata sanguinans* are convex. Furthermore, the shells of *P. compressa* differed morphometrically and thus clustered out from the other species in the PCA and discriminant functions analyses. *Patella miniata miniata* also had a significantly longer X looping of the gut than the other taxa. Further differences were detected in the sperm microstructure. Thus, it was concluded that *P. miniata sanguinans* warrants full specific status. Furthermore, suggestions that *P. miniata* and *P. compressa* may be ecomorphs of the same species are not supported, and both warrant their full specific status. Thus, the '*Patella miniata*' species complex in southern Africa is a closely related monophyletic group of three species, with *P. miniata* and *P. compressa* being more closely related to each other than either one is to *P. sanguinans*.

Key words: patellid, morphology, genetics, allozymes, cladistics, 'gardens'

Acknowledgements

My sincere thanks go to my supervisors, Prof. George Branch (Zoology, University of Cape Town, UCT) and Dr. Barbara Cook (South African Museum, SAM & Zoology, University of Stellenbosch, US) for their support and encouragement throughout this project. Thanks must also go to Prof. Alan Hodgson and his wife, Val, for the sectioning and processing much of the sperm material, as well as for providing me with laboratory space whilst at Rhodes University. Neville Eden (Zoology, UCT) and Miranda Waldron (Electron Microscope Unit, UCT) are thanked for their assistance with preparations of the photographs and micrographs. Further thanks must be extended to Dr. Richard Kilburn (Natal Museum), Dr. David Reid (The Natural History Museum, London) and Dr. Stephen Ridgway (Zoologische Staatssammlung Muenchen) for their assistance in tracking down references and type specimens, and to Dr. Rob Anderson (Sea Fisheries Research Institute, SFRI) and Prof. John Bolton (Botany, UCT) for the identification of the algal samples. Dr. M. Glaubrecht (Museum für Naturkunde, Berlin), Dr. Karl Edlinger (Naturhistorisches Museum, Wien), Dr. Yves Finet (Muséum d'Histoire Naturelle, Genève) and Liz Hoenson (SAM) are also thanked for the loan of various materials. I also thank Prof. George Branch, Dr. Philip Coetzee, Dr. Nancy Teitz, Prof. Arthur Dye, Prof. Theresa Lasiak, Kerry Sink, Bronwen Currie, and Alison and Rico Sakko for assistance with the collection of samples.

Finally, I gratefully acknowledge financial support for this project from the Foundation for Research and Development (FRD), the South African Network for Coastal and Oceanographic Research (SANCOR), and the University of Cape Town.

Chapter 1

General Introduction

Taxonomy

Molluscan taxonomy has historically been based purely on morphological characters, with shell form and radular structure being those most commonly used. However, the large degree of morphological plasticity exhibited by patellid limpets has plagued the taxonomy of this group (Christiaens, 1973; Powell, 1973) and the value of these morphological characters have been questioned (Côrte-Real, Hawkins and Thorpe, 1996b). For example, ratios of shell proportions and radular length within the genus *Patella* reflect different habitats and consequent responses of individuals to environmental stresses (Powell, 1973). However, 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 the status and relationships within molluscan groups. Protein gel electrophoresis is one of the molecular techniques that has been widely used in recent years. This technique allows identification of separate gene pools and is of considerable value in distinguishing species (see Avise, 1975; Hillis and Moritz, 1990). In this way, protein gel electrophoresis has helped to elucidate the taxonomic status of a wide variety of molluscan species including oysters (Hedgecock and Okazaki, 1984), mussels (Skibinski, Cross and Ahmed, 1980; Grant, Cherry and Lombard, 1984), winkles (Heller and Dempster, 1991) and limpets (Cretella *et al.*, 1994; Côrte-Real *et al.*, 1996a,b).

The family Patellidae contains most of the common intertidal limpet species on rocky shores, where they have important influences on community structure (Hawkins, 1981). As a consequence of their abundance, availability and ecological significance, there is extensive literature on the biology and behaviour of patellid limpets (see Branch, 1981). However, the systematics of the Patellidae are often problematic. The genus *Patella* has a widespread distribution, including the Atlantic Ocean, the Mediterranean Sea, the Pacific and the Indian Ocean but cannot be considered cosmopolitan because patellid limpets are absent from the Antarctic, and there is only one species, *Patella mexicana* Broderip and Sowerby, 1829, represented in the whole of the Americas (Powell, 1973). Despite the

widespread nature of this genus, the systematics of the genus *Patella* is still in a state of confusion (Ridgway, 1994). Consensus has yet to be reached on the number of species within the genus, with the two most recent revisions of the genus (Christiaens, 1973; Powell, 1973) differing by as many as eight species. Moreover, these two reviews show marked disagreement about classification above the species level.

In a recent cladistic analysis of most of the world's patellid species based on morphological criteria, Ridgway *et al.* (in press) argue that there are four major clades within the group and that the genus *Patella* (Linnaeus, 1778) *sensu lato* will have to be redefined and divided to recognise their fundamental differences. They distinguish a North Atlantic clade under a more restricted definition of *Patella*, a predominantly western and southern African clade, *Cymbula* (H. & A. Adams, 1854), and a mainly Indo-Pacific and southern African clade, *Scutellastra* (H. & A. Adams, 1854). The fourth clade retains its old generic name, *Helcion* (Montfort, 1810) and is restricted to southern Africa. Lindberg (1998) recognises two major groups within the superfamily Patellidea, the first being *Scutellastra*, and the second including *Patella*, *Cymbula* and *Helcion*. However, because the species complexes I will deal with are well known under the older broad definition of *Patella*, and because the work of Ridgway *et al.* (in press) is not yet published and generally implemented, I will continue to refer to the species collectively under the banner of *Patella sensu lato* throughout this thesis.

Taken in this broad sense, the genus *Patella* shows three centres of diversity, one in the north-eastern Atlantic, one in southern Africa and the third spread more widely over the Indo-Pacific. The genus is most diverse in southern Africa, and the species, despite earlier disagreements (see Krauss, 1848; Reeve, 1854; Bartsch, 1915), are considered to be relatively well characterised in this region. Tomlin and Stephenson (1942) recognised twelve species (*P. argenvillei* Krauss, 1848; *P. barbara* Linnaeus, 1758; *P. cochlear* Born, 1778; *P. granularis* Linnaeus, 1758; *P. longicosta* Lamarck, 1819; *P. tabularis* Krauss, 1848; *P. compressa* Linnaeus, 1758; *P. granatina* Linnaeus, 1758; *P. miniata* Born, 1778; *P. oculus* Born, 1778; *P. sanguinans* Reeve, 1854; and *P. variabilis* Krauss, 1848) as coming from South Africa. The first six of these species fall into the *Scutellastra* clade as defined by Ridgway *et al.* (in press), the next five into the *Cymbula* clade, while the

last-named species, *P. variabilis* has been reassigned by them to the genus *Helcion*, which contains two additional South African species not considered here (*H. dunkeri* Krauss, 1848, and *H. pectunculus* Gmelin, 1791).

Koch (1949) in his review of the South African representatives of the genus *Patella*, divided the genus into two, one group having radulae with the rows of teeth arranged in a V-shape (the *Cymbula* clade), and the other having 'stepped' teeth with the central teeth arranged in a straight line and the laterals set below them (the *Scutellastra* clade plus the *Helcion* species). He recognised the same species as did Tomlin and Stephenson (1942), with the only difference being the omission of *P. sanguinans*. Koch (1949) felt that the variation in colour and ribbing of *P. sanguinans* was not sufficient to maintain it as a separate species, and he therefore synonomised it with *P. miniata*.

In his review, Christiaens (1973) divided the genus *Patella* into five subgenera, two of which had South African representatives. He also recognised eleven South African limpet species, and his classification generally agrees with that of Koch (1949), in that he synonomises *P. sanguinans* with *P. miniata*. However, Christiaens (1973) also mentions *P. pica* Reeve, 1854, as occurring in the tropical Indian Ocean, but he does not record it as coming from South Africa.

In contrast to Christiaens (1973), Powell (1973) listed eight subgenera of *Patella*, six of which contained representatives from South Africa. Powell (1973) agreed with Koch (1949) in that he recognised eleven species as occurring from South Africa, also omitting *P. sanguinans* and *P. pica* from his list.

Kilburn and Rippey (1982) distinguished twelve species of South African limpets, including *P. pica*. However, they mention that *P. miniata* is recognisable as two subspecies, *P. miniata miniata* and *P. miniata sanguinans*, therefore effectively recognising thirteen taxa. All the reviews following Koch (1949) refer to *P. variabilis* as *P. concolor* Krauss, 1848. *Patella variabilis* is invalidated by a prior homonym (Röding, 1798) and *variabilis* was therefore replaced by the earliest available name, *concolor*.

More recently, Robson (1986) added an additional new species of South African limpet, namely *P. aphanes* Robson, 1986, and resurrected *P. obtecta* Krauss, 1848. Thus, according to Jamieson, Hodgson and Bernard (1991), there are fourteen species of *Patella* in southern Africa (*P. aphanes*, *P. argenvillei*, *P. barbara*, *P. cochlear*, *P. compressa*, *P. concolor*, *P. granatina*, *P. granularis*, *P. longicosta*, *P. miniata*, *P. obtecta*, *P. oculus*, *P. pica* and *P. tabularis*). Branch *et al.* (1994) follow the classification of Jamieson *et al.* (1991) except that, like Kilburn and Rippey (1982), they divide *P. miniata* into two subspecies, *P. miniata miniata* and *P. miniata sanguinans*, therefore effectively recognising fifteen taxa. Finally, samples of the Indo-Pacific species, *P. flexuosa* Quoy & Gaimard,1834, have been recorded from northern Zululand in KwaZulu-Natal (Herbert, 1991). Thus, at present, sixteen taxa of limpet belonging to the genus *Patella* are recognised from South African shores.

Key questions to be addressed

In comparison to other regions, the southern African representatives of the genus *Patella* are fairly well characterised. However, there are still some biological and taxonomically interesting questions pertaining to certain of the South African species that warrant study.

Firstly, *Patella granularis* is the most widely distributed of the South African limpet species, yet throughout its range it shows variation in shell size and morphology (Koch, 1949; Kilburn and Rippey, 1982), corresponding to differences in the oceanic environment. Numerous authors have also reported variations in the structure and mineralogy of *P. granularis* shells from the coast of South Africa (MacClintock, 1967; Cohen, 1988; Cohen and Branch, 1992), with the west-coast shells differing from those from the south and east coasts because they possess an additional (inner) shell layer and a thicker outer layer.

- Based on the shell structural differences, do the west-coast individuals of *P. granularis* form a morphologically and/or genetically distinct group from the south and east coast individuals?

Secondly, *Patella barbara* also has a widespread distribution, and according to Koch (1949) it exhibits the greatest differences in shell shape and coloration of all the South African limpet species. Furthermore, *P. barbara* has been shown to 'garden' algal resources on the south and east coasts of South Africa (Branch *et al.*, 1992), whereas on the west coast they have no 'gardens'. Thus, there is a difference in behaviour between west-coast individuals and those from the east and west coasts of South Africa.

- Are the shell variation and behavioural differences between populations of *P. barbara* mirrored by other morphological and/or genetic differences?

Finally, questions arise about the relationships of the closely-related taxa that fall into a '*P. miniata*' complex. *Patella miniata*, although showing marked colour gradations around the coast (Koch, 1949), is currently recognised as a valid species on South African shores. *Patella sanguinans*, on the other hand, was recognised by Tomlin and Stephenson (1942) but since then it has been dismissed and included as a synonym of *P. miniata* or reduced to subspecific level. Koch (1949) considered that the variation in the sculpturing and the coloration of the shells of *P. sanguinans* was not sufficient to distinguish it from *P. miniata*, because similar variations have been shown to occur in other species of South African limpets. However, Kilburn and Rippey (1982) and Branch *et al.* (1994), although not separating *P. sanguinans* as a valid species, recognise the differences recorded by Tomlin and Stephenson (1942) and divide *P. miniata* into two subspecies; *P. miniata miniata* and *P. miniata sanguinans*. Furthermore, in a recent paper on a cladistic phylogeny of the family Patellidae, Ridgway *et al.* (in press) failed to include *P. sanguinans* as a separate species, since they considered it identical to *P. miniata* in the morphological characters examined in their study, differing only in the size and coloration of the shell.

- Does *P. sanguinans* warrant specific status, or is it merely subspecific or synonymous within *P. miniata*?

Patella compressa is another member of the '*P. miniata*' complex. Although generally recognised as a valid species, it was considered by Pilsbry (1891) as being conspecific to *P. miniata*. Pilsbry (1891) stated that the species develops into the *compressa* form on kelp, and into the *miniata* form on rocks. Pilsbry's (1891) suggestion has not gained general acceptance and has been dismissed by numerous authors (Koch, 1949; Christiaens, 1973; Powell, 1973). However, Ridgway *et al.* (in press) also suggest that *P. miniata* and *P. compressa* may prove to be ecomorphs of the same species (living on rocks and kelp respectively).

- Are *P. miniata* and *P. compressa* ecomorphs of a single species, or valid and separate species?

A last constituent of the '*P. miniata*' complex has caused confusion in recent years. On the south coast of South Africa, *P. miniata* is readily recognisable and has an attractive pink shell with fine radiations and a low profile. On the west coast there exists a variety that is recognisably 'different', with a tall shell that is tinged blue. Moving further north into Namibia, this variety becomes even more distinctive. This variety has been tentatively identified by R. Kilburn (pers. comm.) as a form of *P. safiana* Lamarck, 1819, (a species which does occur in West Africa from Angola northwards). It has also been tentatively named *P. adansonii* Dunker, 1853, by Ridgway *et al.* (in press), because of its resemblance to the type of this species illustrated by Powell (1973). In this thesis, I have referred to this variety as *P.* cf. *miniata*.

- Is *P.* cf. *miniata* distinct from *P. miniata*, and what is the validity of the name *P. adansonii*?

In view of these interesting questions and the taxonomic confusion still surrounding certain of the South African patellid limpets, together with the limitations of traditional morphology to resolve taxonomic problems, it was decided to combine both morphological and biochemical (protein gel electrophoresis) methodologies, in an attempt to solve these questions, and to clarify the number of species in the genus *Patella* in South Africa. Very little published work integrates both morphological and genetic characters of patellid

limpets, although recent work has been carried out in the northern hemisphere (Cretella *et al.*, 1994, Côrte-Real *et al.*, 1996a,b). Thus, the work presented in this thesis is the first time that protein gel electrophoresis has been conducted on patellid limpets in southern Africa. Furthermore, it is the also the first time that morphological and genetic techniques have been integrated in the same study on patellid limpets in southern Africa.

The key questions outlined above are dealt with, either separately or in parts, in each of the six chapters of this thesis. Brief outlines of the research surrounding each of the chapters are given below.

Thesis outline

Chapter 1

General introduction.

Chapter 2

This chapter examines the morphological and genetic differentiation of *P. granularis* along the coast of southern Africa. Shell characters, radular morphology, gut loop coiling and sperm microstructure were integrated with protein gel electrophoresis to quantify coast-wide differentiation between thirteen populations of the species thus far identified as *P. granularis*.

Chapter 3

In Chapter 2 I concluded that the east-coast populations of "*P. granularis*" are sufficiently different from the south and west coast populations to warrant the recognition of two separate species. In this chapter I present a re-description of *P. natalensis* Krauss, 1848, from the east coast of South Africa, together with a detailed comparison of *P. natalensis* with *P. granularis*.

Chapter 4

This chapter explores the morphological and genetic differentiation of *P. barbara* along the coast of South Africa. Shell characters, radular morphology, gut loop coiling and sperm microstructure were integrated with protein gel electrophoresis to quantify differentiation between eight populations. In addition, note is made of variations of the behaviour of *P. barbara* in terms of the 'gardening' of algal resources around the coast. Mention is also made of the unusually-shaped variety of *P. barbara* which is found on the ascidian *Pyura stolonifera* in False Bay.

Chapter 5

A re-examination of the taxonomy of the '*Patella miniata*' species-complex is presented in this chapter. Morphological (shell characters, radular morphology, gut looping and sperm microstructure), genetic (protein gel electrophoresis), and a phylogenetic analysis are used to resolve the taxonomic confusion surrounding *P.* cf. *miniata* (referred to as *P. adansonii* by some), *P. miniata*, *P. sanguinans*, and *P. compressa*. In addition, a population of *P. safiana* from Angola was included in the analyses.

Chapter 6

This chapter is the final synthesis, briefly summing up the results from the above chapters.

The thesis is set out in such a way that chapters 2 - 5 have been written as discrete units, modifications of which have been, or will be, submitted for publication in the following international scientific journals:

Chapter 2 in the *Journal of Zoology, London*
Chapter 3 in the *Journal of Molluscan Studies*
Chapter 4 in the *Journal of the Marine Biological Association of the United Kingdom*
Chapter 5 in *Marine Biology*

Chapter 2

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

INTRODUCTION

The patellid limpet *Patella granularis* Linnaeus 1758, as currently recognised, 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 and Penrith, 1973; Powell, 1973). Throughout this range *P. granularis* shows variation in shell size and morphology (Koch, 1949; Kilburn and 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 and 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 and 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 and 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, morphological variability exhibited by limpets has created difficulties in the taxonomy of this group (Powell, 1973) and the value of these morphological characters is therefore questionable (Côrte-Real *et al.*, 1996b). 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 are darker in appearance than those from the west coast (Kilburn and Rippey, 1982), has led to numerous uncertainties about 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 and Dempster, 1991; Boulding, Buckland-Nicks and Van Alstyne, 1993), very little published work integrates morphological and genetic characters of patellid limpets (Côrte-Real *et al.*, 1996a).

Given the contrast in shell structure between the west-coast individuals and those of the south and east coasts, it was initially hypothesised 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 and Bernard, 1991; Kool, 1993). However, certain 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 this chapter, 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 group morphologically and/or genetically distinct 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. 2.1). The four biogeographic provinces mentioned follow those proposed by Emmanuel *et al.* (1992), but for simplicity, the two cool-temperate provinces are hereafter combined and termed the 'west coast'. Individuals used for morphological analyses were preserved in 70% alcohol. For genetic analyses, live animals were transported to the laboratory where they were stored in an ultra deep-freeze (at -80°C) for later electrophoretic analysis.

Morphological analyses

Shell texture and pedal coloration: The pigmentation of the nodules on the costae (Fig. 2.2) was recorded for all sample sites. The number of costae on the shells (Fig. 2.2) was counted for 15 individuals from all but 2 of the sample sites. Shells from Swakopmund and Groen River were excluded from this part of the analysis because the costae were not

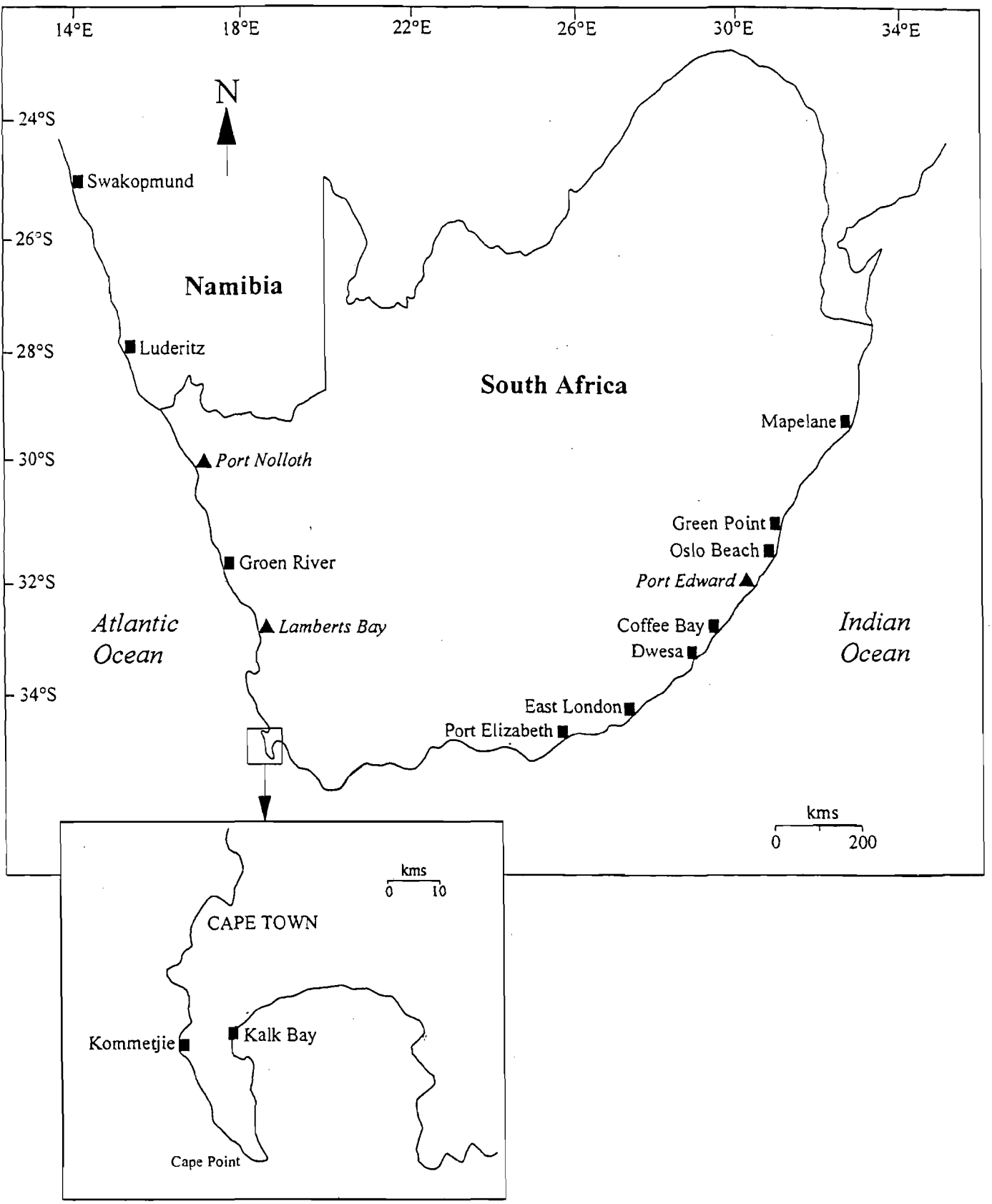


Figure 2.1 Map of southern Africa showing the 12 sites (■) at which *Patella granularis* was sampled, supplemented with museum material from three sites (▲).

always clearly defined due to erosion, 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 nodules on the costae was also scored. The nodules were counted on the left and right anterior-most and posterior-most costae (Fig. 2.2). 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.

Shell morphometrics: Shell morphometrics were obtained by measuring the shells of 624 individuals from the 12 sample sites (Fig. 2.1), as well as from three additional sites from the dry mollusc collection at the South African Museum (SAM). These additional sites (also shown in Fig. 2.1) 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 0.01mm) 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 the use of ratios in regression analysis is flawed because the effect of size has not been removed from the shape variable (Atchley, Gaskins and Anderson, 1976), although ratios can be regressed on size and the effect of size eliminated as a co-variate. It was thus decided not to use ratios but rather to use the

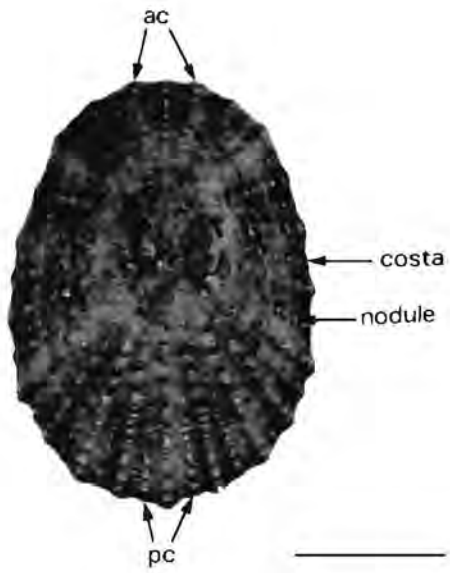


Figure 2.2 Dorsal view of the shell of *Patella granularis* showing the costae, nodules and the costae on which the nodules were counted. ac, anterior costae, and pc, posterior costae on which nodules were counted. Scale bar: 10mm.

original measurements for the multivariate statistics. 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 two or more groups based on the variables used. A set of discriminant functions is produced by which each specimen is assigned to one of the groups on which the analysis is based (Côrte-Real *et al.*, 1996a). 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 two to five 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 mineralisation 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 minutes. They were then transferred to 70% alcohol and ultrasonicated for a further 60 seconds, 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. 2.3) 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 samples sizes, the data from the 5 populations were pooled into the 3 coastal zones outlined above.

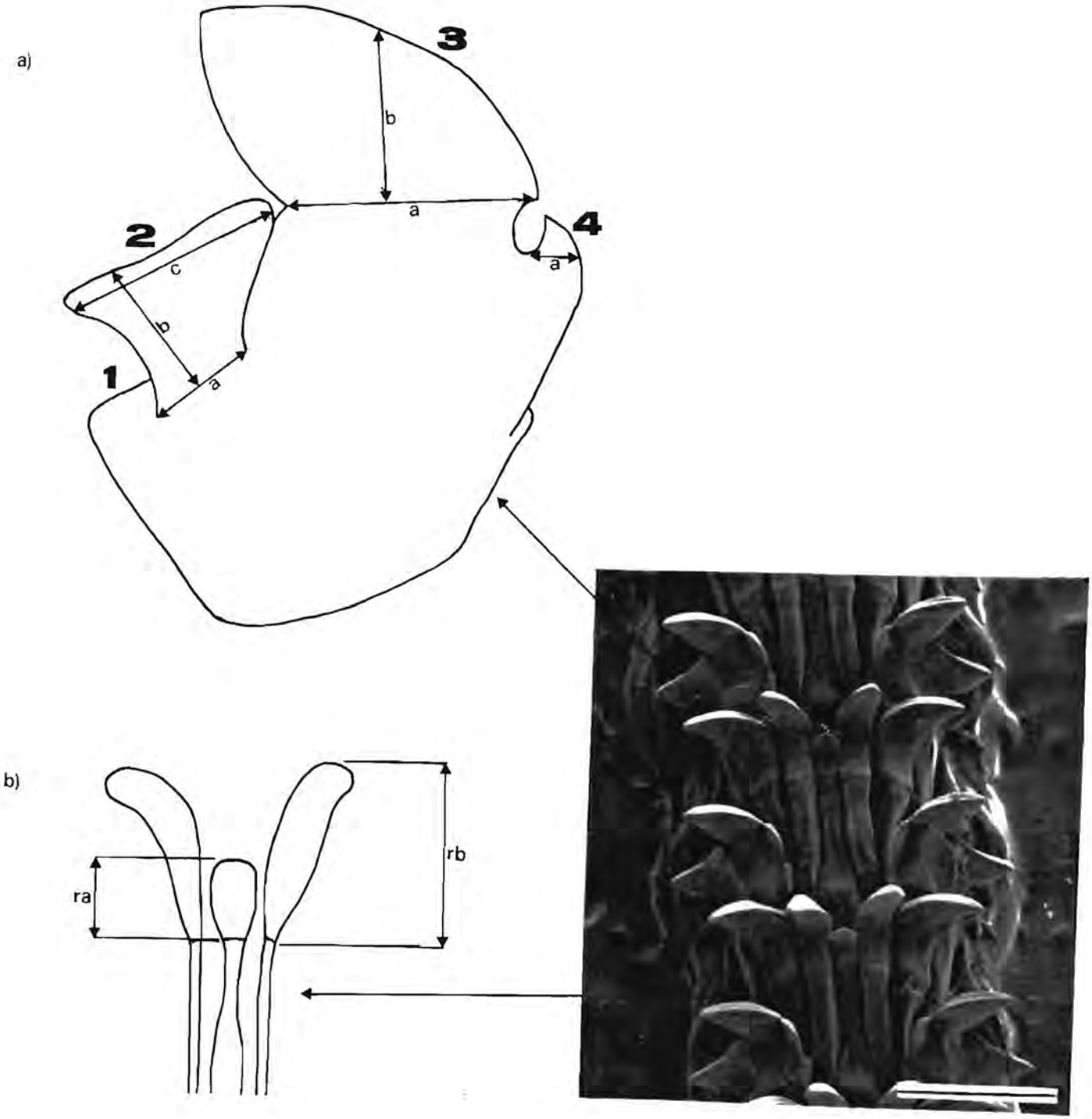


Figure 2.3 Scanning electron micrograph of the radular morphology of *Patella granularis* with diagrammatic representations of (a) the pluricuspid tooth, and (b) the rachidian and first lateral teeth, showing the dimensions measured. See Methods for identities of the measurements. Scale bar: 200μm.

A Kruskal-Wallis test and the Wilcoxon two-sample test were performed based on the methods outlined by Dunn (1964) in order to test for significant differences between the three 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. 2.4. Four measurements (Fig. 2.4) were taken from the gut loops of each individual and three 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 two-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 two sites from each of the three coastal regions. Small portions of the testis of five 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 five 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.

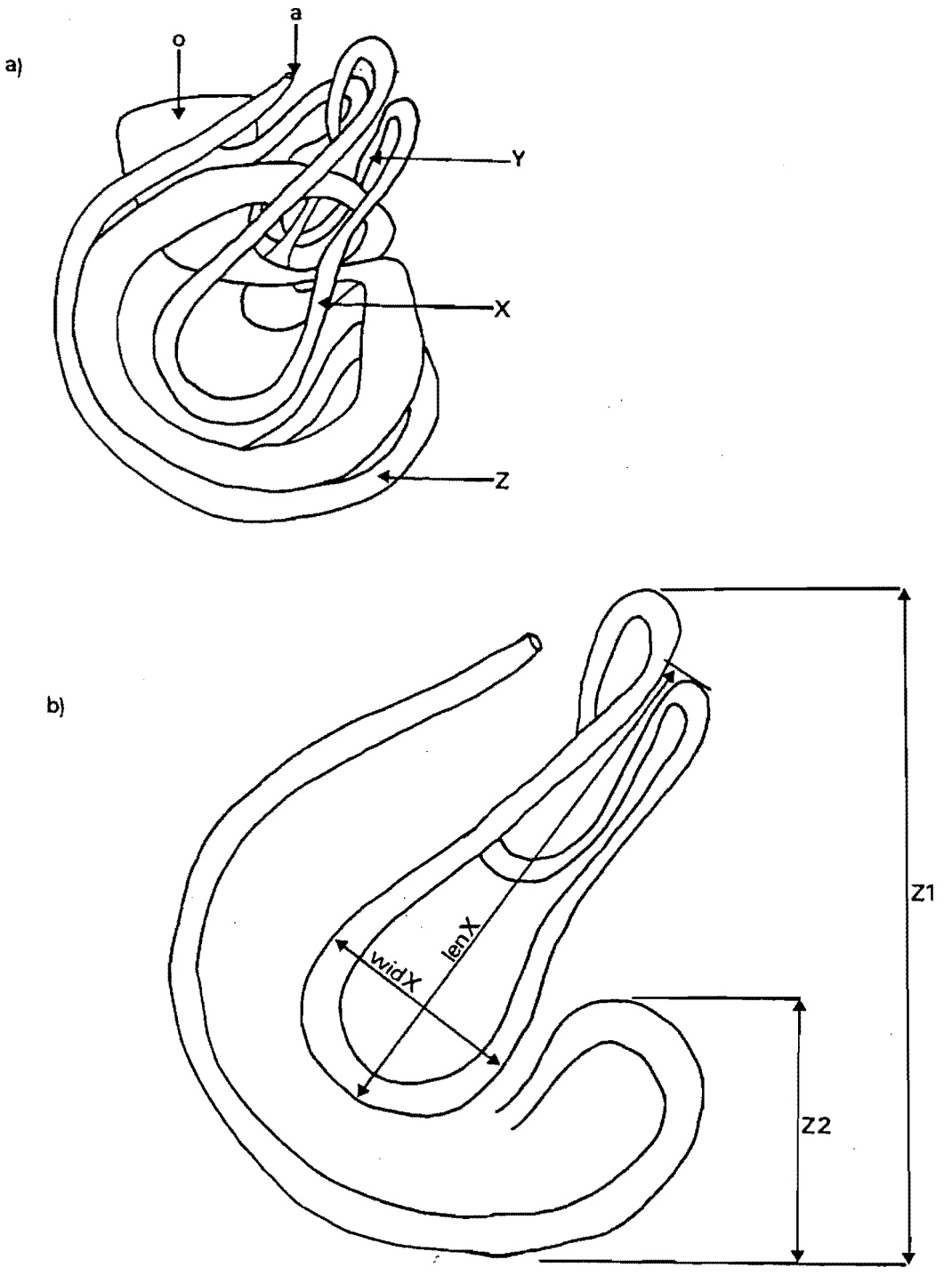


Figure 2.4 Diagrammatic representation of the gut loops of *Patella granularis* after complete dissection from the digestive gland showing (a) the relative positions of the X, Y and Z loops, and (b) the dimensions of the X and Z loops measured. See Methods for identities of the measurements. a, anus; o, oesophagus.

The samples fixed in 2.5% gluteraldehyde were washed for 30 minutes in 0.2M sodium cacodylate buffer (pH 7.2) and post-fixed for 90 minutes in 1% osmium tetroxide in 0.2M 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 minutes and lead citrate for 3 minutes. 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. 2.5). 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 and Hopkinson, 1976). Sample sizes are shown in Table 2.5. Foot muscle and mantle tissue were prepared from each individual. Tissue samples were roughly minced using a scalpel and then homogenised in 0.01M 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 minutes. Gels were prepared with 12.5% hydrolysed potato starch. The following buffer systems were used: (a) Tris-citrate-lithium hydroxide-borate buffer, pH 8.0 (Ridgeway, Sherburne and Lewis, 1970), (b) Tris-borate-EDTA buffer, pH 8.6 (Markert and Faulhaber, 1965) and (c) Tris-citrate buffer, pH 6.9 (Whitt, 1970). All gels were run for between 4-5 h at a constant current of 30 mA. Gels were sliced into 4 slices, which were then stained for different enzymes using specific histochemical stains (Shaw and Prasad, 1970; Harris and Hopkinson, 1976). The enzymes assayed, the buffer systems, and the tissue used are listed in Table 2.1.

The electrophoretic data were analysed using the programme BIOSYS-1 Release 1.7 (Swofford and Selander, 1981). Allele and genotype frequencies were calculated. Deviations of genotype frequencies from Hardy-Weinberg equilibrium were measured

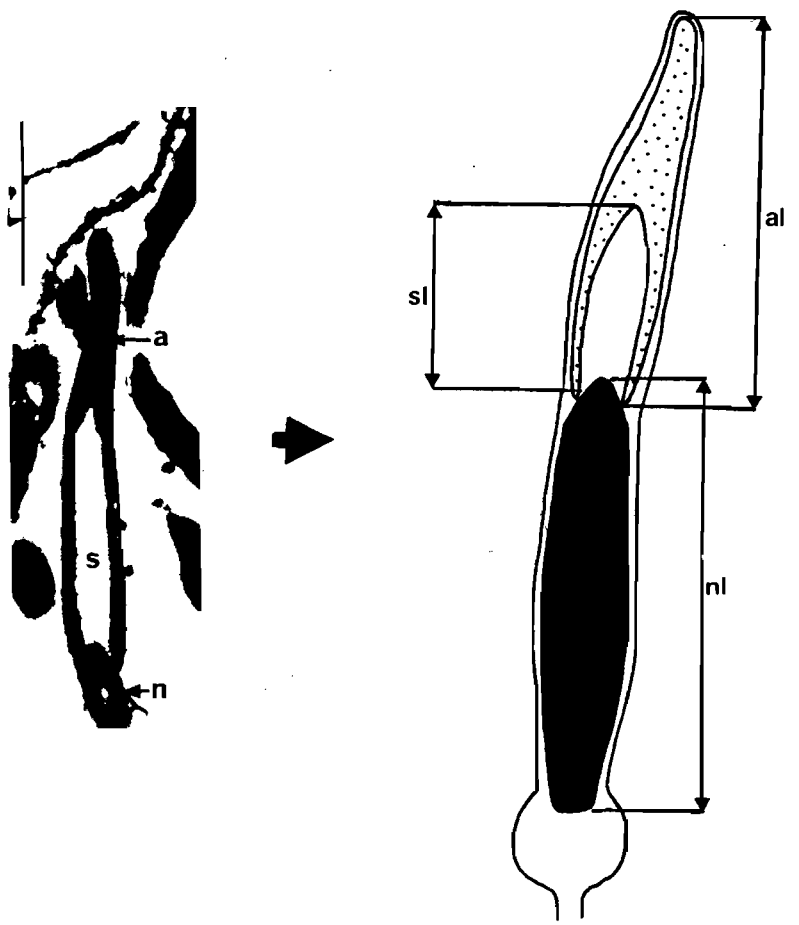


Figure 2.5 Mid-longitudinal TEM section through the spermatozoa of *Patella granularis* with a diagrammatic representation of the dimensions measured. a, acrosome; al, length of acrosome; n, nucleus; nl, length of nucleus; s, subacrosomal space; sl, length of subacrosomal space. Scale bar: 1μm.

using Levene's (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).

Table 2.1 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 ME-2	(b) (b)	Mantle 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 MDH-2	(c) (c)	Mantle Mantle
Isocitrate dehydrogenase (IDH)	1.1.1.42	IDH-2	(c)	Mantle
Aspartate amino transferase (GOT)	2.6.1.1	GOT-1	(c)	Mantle

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 2.2). The shells from Coffee Bay were mixed, 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 (ANOVA, $p<0.05$). 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 a creamy-orange undersides to 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.

Table 2.2 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
Foot colour : side	grey	creamy-grey	grey	cream	creamy with grey flecks	creamy with grey flecks	creamy with grey flecks	creamy-grey	grey	grey	grey	grey	grey

Shell morphometrics: Examination of shell lengths (Fig. 2.6) shows that size tends to decline from west to east (Table 2.3). Figure 2.7 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 accounting for only 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 6 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 morphometrics differentiates between the populations from the west, south and east coasts.

Radular morphology: No difference in radular 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 unmineralised 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 unicuspid teeth. The pluricuspid tooth has four cusps, with cusp 3 being pointed and larger than the other cusps. Table 2.4 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

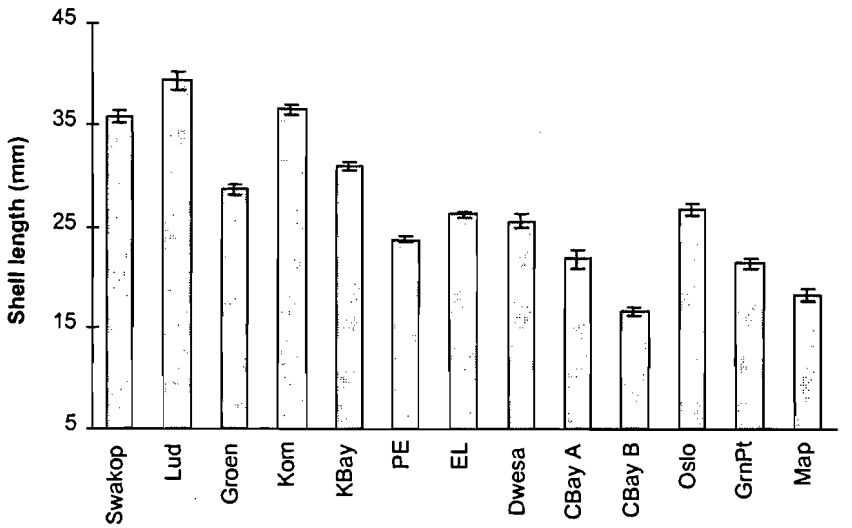


Figure 2.6 Mean (±SE) of the lengths of the shells of *Patella granularis* from the 13 populations along the southern African coast. Populations are arranged in geographical order around the coast from west to east.

Table 2.3 Mean and standard error of the shell dimensions measured from 13 populations of *Patella granularis*. See Methods for explanation of abbreviations.

	Population	SL (mm)	SW (mm)	SH (mm)	WT (g)	VOL (ml)
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

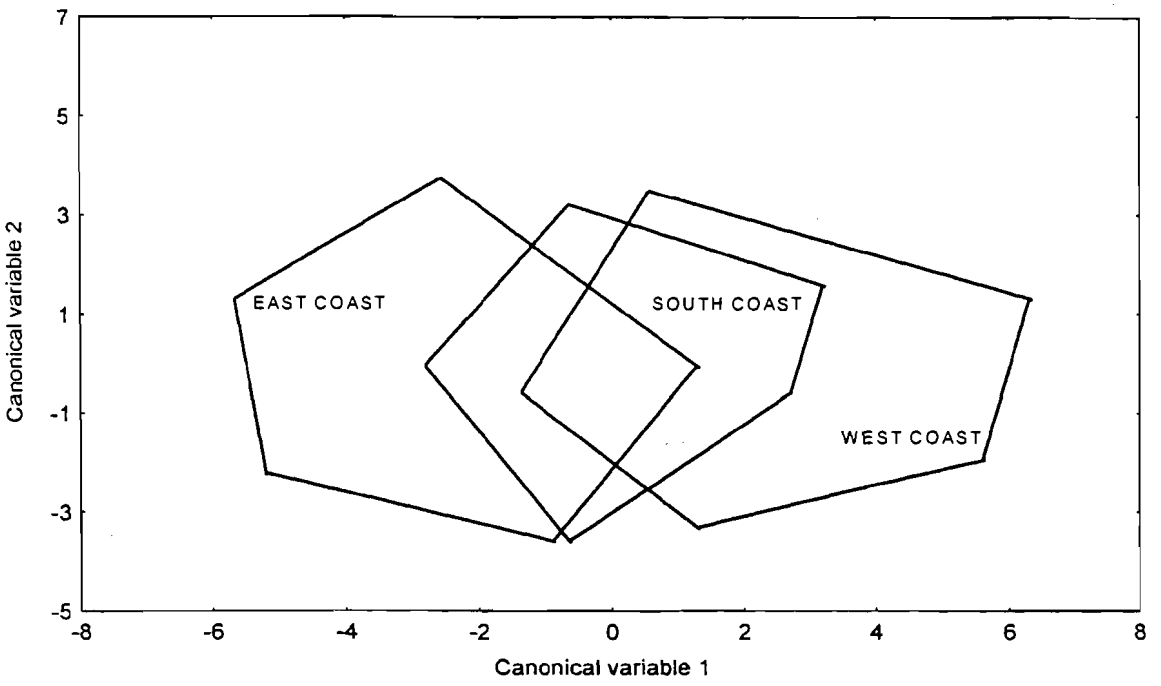


Figure 2.7 Plot of the first two canonical variables for the west, south and east coast populations of *Patella granularis*. The outlines are convex hulls surrounding individuals from the three coastal regions.

Table 2.4 Mean and ranges of the five measurements taken from the radulae 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

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 (n = 5) make comparison between the populations very difficult but the results do suggest that the Green Point population differs from the other populations, even if not in a manner that can be used for unequivocal diagnosis.

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. 2.8. The length of the Z loop (Fig. 2.8a) shows that individuals from Coffee Bay B, Oslo Beach, Green Point and Mapelane had relatively shorter Z loops than the other nine populations, differing significantly from Swakopmund, Luderitz, Port Elizabeth, East London, Dwesa and Coffee Bay A (Kruskal-Wallis, $p<0.05$). 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. 2.8b) 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 of the X loop relative to its length (Fig. 2.8c) 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, most of the patterns and loops of the mid and hindgut remained fairly constant throughout the populations, or yielded differences that were of no assistance in distinguishing populations between regions. The solitary exception was the relatively shorter Z loop in the four northern-most east-coast populations (Fig. 2.8a).

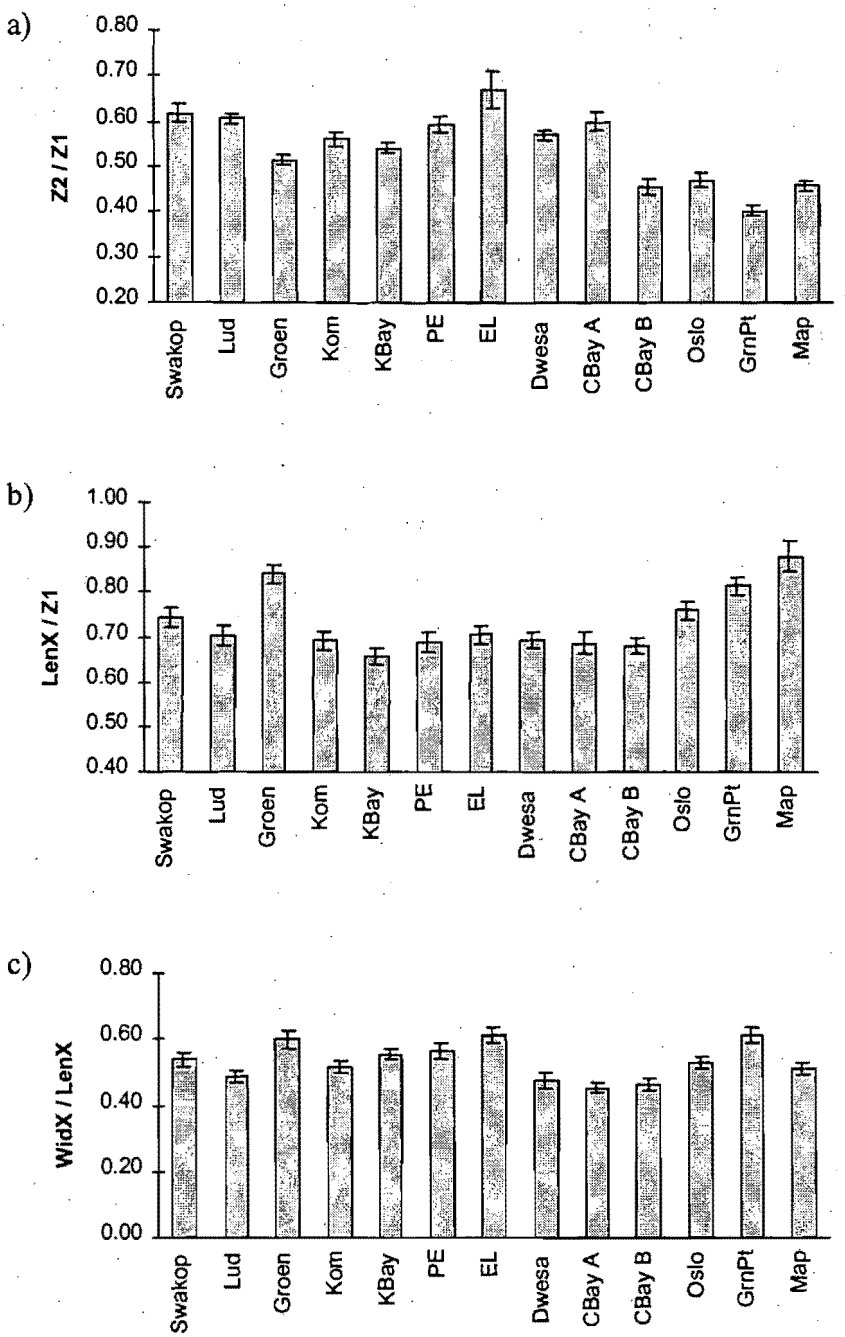


Figure 2.8 Gut loop measurements (mean ± SE) for the 13 populations of *Patella granularis*. See Figure 2.4 for interpretation of abbreviations.

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 *et al.*, 1996). Figure 2.9a 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 shorter (ANOVA, $p<0.05$) than the Kalk Bay population, but there was no clear-cut pattern distinguishing the different coastal regions. Figure 2.9b 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 2.9c and 2.9d 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 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 lengths of the acrosome and subacrosomal space 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 2.5. 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 2.5. 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).

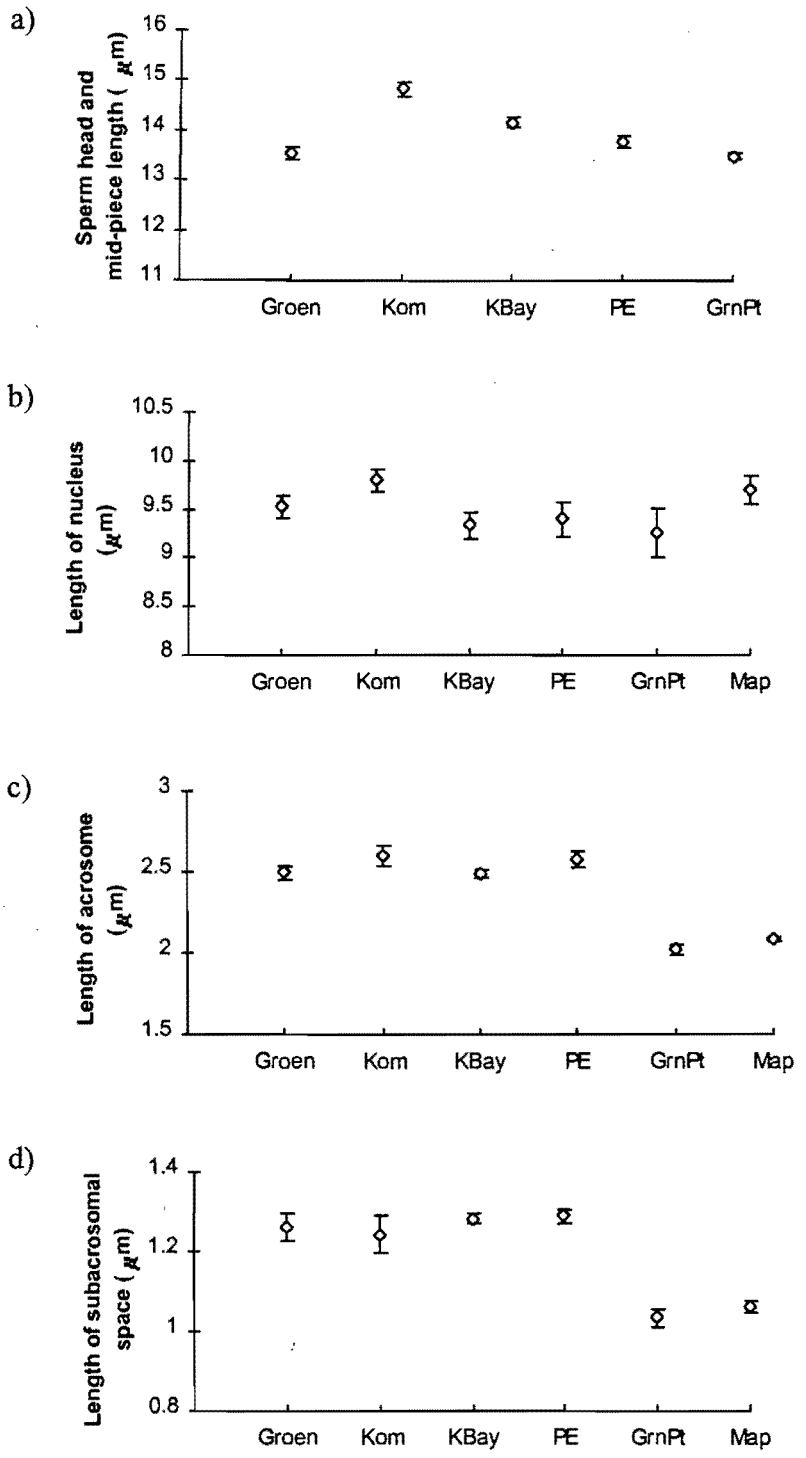


Figure 2.9 Sperm microstructure measurements (mean ± SE) for the six populations of *Patella granularis* examined.

Table 2.5 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
ARK-2													
(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

Table 2.5 (contd.)

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	-	-	-	-	-	-	-	-

Tests for Hardy-Weinberg equilibrium (using Levene's (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 in all cases deviations occurred 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. 2.10) of Nei's (1978) unbiased genetic identities (*I*) between all pairs of populations (Table 2.6) 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 hypothesised. This finding does not coincide with the shell structural differences reported by Cohen (1988) and Cohen and 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

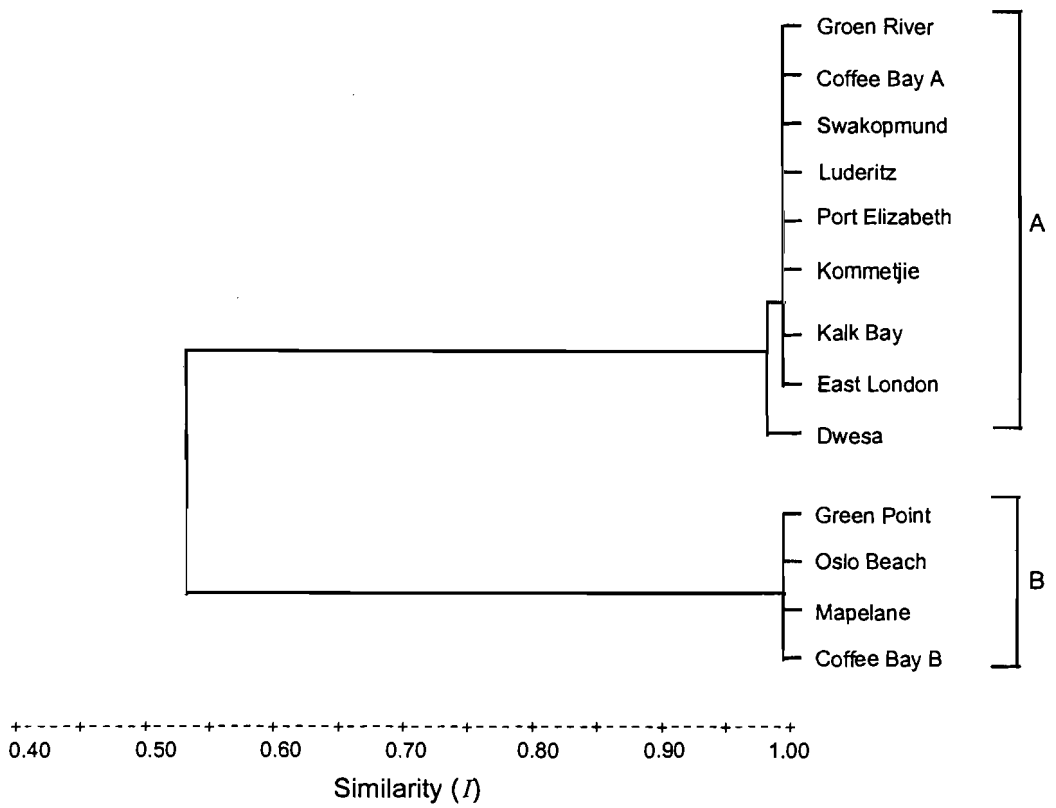


Figure 2.10 UPGMA dendrogram derived from Nei's (1978) genetic identity based on 16 loci for 13 populations of *Patella granularis*.

Table 2.6 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	*****

presence of a calcitic *m* - 2 layer distinguishes all west-coast populations from those elsewhere. However, there is no evidence at all suggesting that west-coast populations are genetically separable from those of the south coast (Fig. 2.10). Rather, the most striking result obtained in this chapter 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, Kommetjie, 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. 2.10).

The genetic identity value (Nei, 1978) at which 'group A' and 'group B' separated (Fig. 2.10) 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.* (1996a) 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 and Dempster, 1991).

However, the most convincing evidence obtained in this study is the co-occurrence of the two genetically distinct forms at Coffee Bay (Table 2.5). The failure to find a single individual from either genetic grouping possessing the allele of the other at the diagnostic

loci, indicates an absence of gene flow between the two forms, therefore implying reproductive isolation. In their review on species concepts, McKitrick and 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 emphasises 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 fact that the two genetic forms co-exist at Coffee Bay (Table 2.5) provides clear evidence that there is no gene flow between the two groupings and therefore 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 and 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 and Hewiston, 1994). *P. granularis* is a broadcast spawner, and upwelling off Mbashe may have provided sufficient a 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 genetic 'group A' have nodules which are whitish in colour whereas the shells from 'group B' have nodules

which are black in colour (Table 2.2). 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 chapter the colour of the underside and the side of the foot showed no diagnostic features (Table 2.2). The comparison of the radulae also revealed no differences, with all the populations examined having the same overall radular structure and relative dimensions (Table 2.4). 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 utilise their radular apparatus in similar ways (Hawkins *et al.*, 1989).

The pattern of the loops of the mid and hindgut have 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. 2.8), 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 and Bernard, 1989; Hodgson and 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. 2.9) 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. 2.6). A strong intertidal primary productivity gradient on rocky shores exists

around the coast of South Africa (Bustamante *et al.*, 1995), with the production being the 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 and Hockey, 1988; Branch *et al.*, 1987). Furthermore, the size of *P. granularis* is influenced by the density of barnacles on the shore (Branch, 1976). The fact that body size is related to environmental factors makes it very unlikely that size has any taxonomic value in distinguishing populations of *P. granularis*.

The discriminant functions analysis of the shell morphometric data revealed no distinctive groupings (Fig. 2.7), with only 57.4% chance of correctly re-assigning 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 and 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 2.2). Thus, the variation in shell morphometrics between the populations can probably be attributed to differing environmental influences between the sample sites.

Thus, the results reveal that, despite striking morphological similarities between populations of the limpet currently lumped under the name "*Patella granularis*", the four northern-most 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, recognised both *P. natalensis* and *P. echinulata*. Turton's (1932) account also recognised *P. natalensis* but he placed *P. echinulata* as a subspecies of *P. natalensis*. However, Tomlin and 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 and 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.

However, this chapter conclusively shows that the populations of "*P. granularis*" from the four northern-most sites on the east coast of South Africa are not in fact this species, but rather represent 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. *Patella 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 Chapter 3.

Chapter 3

Re-description of *Patella natalensis* Krauss, 1848 (Gastropoda: Patellidae) and a comparison with related species

INTRODUCTION

Patellid limpets are morphologically plastic and this has complicated the taxonomy of this group in the past (Powell, 1973). Even *Patella granularis* Linnaeus, 1758, the most common and widely distributed of the southern African species varies in shell size and morphology throughout its range (Koch, 1949; Kilburn and Rippey, 1982; Bustamante *et al.*, 1995) and this has led to numerous uncertainties with regard to its taxonomy (see Powell, 1973). It was this morphological variation that led to the examination of the morphological and genetic differentiation of *P. granularis* along the southern Africa coast (Chapter 2). This resulted in the identification of two distinct genetic groups, with individuals from the east coast of South Africa being distinct from those from the south and west coasts. These two groups were also found to be reproductively isolated when occurring in sympatry. The colour of the nodules on the costae of the shells also showed differences, with the east-coast shells having black nodules whereas those from the south and west coasts had nodules of a white coloration. The east-coast individuals also differed in gut morphology and sperm microstructure. These genetic and morphological differences were considered sufficient to warrant the recognition of two separate species (Chapter 2).

After examination of the holotype and the original description of *Patella granularis*, animals from the south and west coasts were assigned to this species. Thus, this chapter outlines the reasons for reassigning the east-coast specimens as a different species, which has involved unravelling a complex taxonomy, and provides a fresh re-description of this species.

TAXONOMY

In 1848 Krauss described several species of patellid limpets of which two are relevant to the present analysis. The first, *Patella echinulata* Krauss, 1848, putatively came from Table Bay on the west coast of South Africa. The second, *Patella natalensis* Krauss, 1848, came from Natal (now known as KwaZulu-Natal) on the east coast of South Africa. According to Krauss (1848), both *P. echinulata* and *P. natalensis* had black nodules on the costae of the shell. Unfortunately, according to Janus (1961) and R. Kilburn (pers. comm.) the Krauss type specimens were 'lost' during World War II, therefore making it impossible to examine the type material of these taxa. Thus, it is only possible to follow Krauss's (1848) published descriptions and illustrations of these two species. Based on this information, both *P. echinulata* and *P. natalensis* resemble each other and the east-coast form detected in Chapter 2. However, *P. echinulata* was reported as being found in Table Bay, which does not coincide with the distribution recorded for the east-coast variant in Chapter 2. I suspect that a mistake occurred in recording the type locality of *P. echinulata*, as has happened with other Krauss type localities in South Africa (R. Kilburn, pers. comm.). Furthermore, I believe that *P. echinulata* is in fact the same species as *P. natalensis* (Krauss, 1848).

In 1853, Dunker erroneously synonomised both *P. natalensis* and *P. echinulata* with *P. nigrosquamosa* Dunker, 1846. This synonymy was based on the fact that Philippi (1849) reported finding a southern African variety of *P. nigrosquamosa*. However, judging from his illustrations, Philippi's (1849) material was in reality *P. miliaris*, which occurs on the west coast of Africa. Moreover, the true *P. nigrosquamosa* is in reality a synonym of *P. rustica* Linnaeus, 1758, which ranges from the Atlantic coast of south west France, through Portugal, Spain and the Mediterranean (Powell, 1973) and therefore is not a South African taxon.

On the other hand, Koch (1949) synonomised *P. natalensis* and *P. echinulata* with *P. granularis*. However, a year later, Nicklès (1950) recognised *P. natalensis* as a species, reporting it as coming from Angola and South Africa. It therefore seems probable that Nicklès (1950) misidentified *P. miliaris* from Angola as *P. natalensis*, as both species have black

nodules on their shells. In the more recent accounts of patellid limpet taxonomy, both Powell (1973) and Christiaens (1973) synonomise *P. natalensis* and *P. echinulata* with *P. granularis*. Curiously, Christiaens (1973) also synonomises *P. natalensis* with *P. nigrosquamosa*.

Thus, although *P. natalensis* has been recognised as a separate species in the past (Krauss, 1848; Nicklès, 1950), it has generally been synonomised with *P. granularis*, with the *P. nigrosquamosa* synonym not being valid because *P. nigrosquamosa* (= *P. rustica*) is not a South African taxon. This synonymy of *P. natalensis* with *P. granularis* has generally been accepted in ecological work in the region, and therefore these studies mention the presence of only *P. granularis* (Stephenson, 1937; Branch, 1981; Bustamante *et al.*, 1995). However, it is now clear that the east-coast animals in Chapter 2 are not *P. granularis* but rather resemble *P. natalensis*. Thus there is a need to recognise the validity of *P. natalensis*, to provide a full description and, particularly, to clarify its diagnostic features and distinguish it from *P. granularis*.

MATERIALS AND METHODS

Samples of *Patella granularis* were collected from intertidal rocky shores at 9 localities along the coast of southern African from Swakopmund in Namibia to Coffee Bay on the Transkei coast of South Africa. Samples of the east-coast counterpart of this species were collected intertidally at 4 localities from Coffee Bay to Mapelane on the KwaZulu-Natal coast of South Africa. All animals were transported live to the University of Cape Town. Spermatozoa were obtained from the testes of five mature, live males from each of six of the sites. The sperm was fixed in 4% formalin or 2.5% gluteraldehyde in filtered sea water. In addition, whole specimens of about 15 to 20 individuals from each of the sites were fixed in 70% ethanol and transported to the laboratory for morphological analysis.

The pigmentation of the nodules on the costae, the number of costae on the shells and the number of nodules on the anterior and posterior costae were scored or counted for 15 individuals from all but two of the sample sites (Chapter 2). Ten individuals from each site

were dissected to record the colour of the soft-part morphology and to examine the patterns of the loops of the mid and hindgut (after 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 relative to the X loop, and the length of the Z loop relative to the visceral mass. The terminology and measurement of these loops follows Chapter 2.

The anterior sections of radulae were dissected from two to five individuals from five of the sample sites and examined using scanning electron microscopy (see Chapter 2 for details). The terminology used for the description of the radula follows that of Koch (1949) and Powell (1973).

The fixed sperm samples were processed for light and transmission electron microscopy (TEM) at the Department of Zoology and Entomology at Rhodes University. 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 five individuals from each population. All measurements were carried out under oil immersion using a Nikon Filar micrometer eyepiece. The samples fixed in 2.5% gluteraldehyde were prepared for TEM measurements (see Chapter 2 for details).

Shell morphology and measurements were recorded for all specimens collected at the 12 sampling sites as well as for individuals from 3 additional sites, which are housed in the South African Museum (SAM A52929, SAM A52930, SAM A53165). Specimens from two of these additional sites were *P. granularis*; those from the third site were the hitherto unrecognised species. The protocol and measurements follow those outlined in Chapter 2. The morphometric data were log transformed and analysed by stepwise discriminant functions analysis (Statistica for Windows Release 5.1, StatSoft Inc., 1996). In addition, scatterplots of SL, SW and SH were drawn using Statistica for Windows Release 5.1, StatSoft Inc., 1996.

SYSTEMATIC DESCRIPTION

Family: Patellidae Rafinesque, 1815

Patella natalensis Krauss, 1848

Neotype: SAM A54196. SL = 15.38 mm, SW = 10.63 mm, SH = 6.05 mm. Type locality: Green Point, KwaZulu-Natal, South Africa. On rocks; coll. K. Sink, June 1996.

Description: Shell (Fig 3.1) - ovate, slightly narrowed anteriorly. Conical with apex slightly anterior of the centre. Sculpture of strong regular costae with pronounced black nodules on the costae. External coloration: light brown with black nodules on the costae and white flashes radiating from the apex. Internal coloration: bluish white, often with light brown radiations and with thin dark brown to black marginal border, and a bluish-white to brown spatula in the centre.

Animal - underside of the foot ranging from light to dark grey. Sides of foot consistently dark grey in coloration. Head dark grey above with a creamy-grey mouth below. Tentacles on either side of the head dark grey on the upperside and light grey on the lower side. Mantle tissue cream with a mottled black marginal border. Upper loop (X loop) of the gut coiling neither clockwise nor anti-clockwise. Y loop less than 30% of the length of the X loop. Z loop about 45% of the length of the visceral mass.

Radula - Typical docoglossate radula described by the formula 3 + 1 + (2 + 1 + 2) + 1 + 3. Consists of three pairs of unmineralised marginal teeth, three pairs of lateral teeth and a single, small, central rachidian tooth. The lateral teeth are divided into a pair of large pluricuspid teeth and two pairs of smaller unicuspid teeth. The unicuspid teeth form a straight line, with the pluricuspid teeth lying below them, thus forming a 'stepped' rather than an inverted 'V' shape. The pluricuspid tooth has four cusps (numbered from the outside to the centre of the radula), with cusp 3 being pointed and larger than cusps 1, 2 and 4.

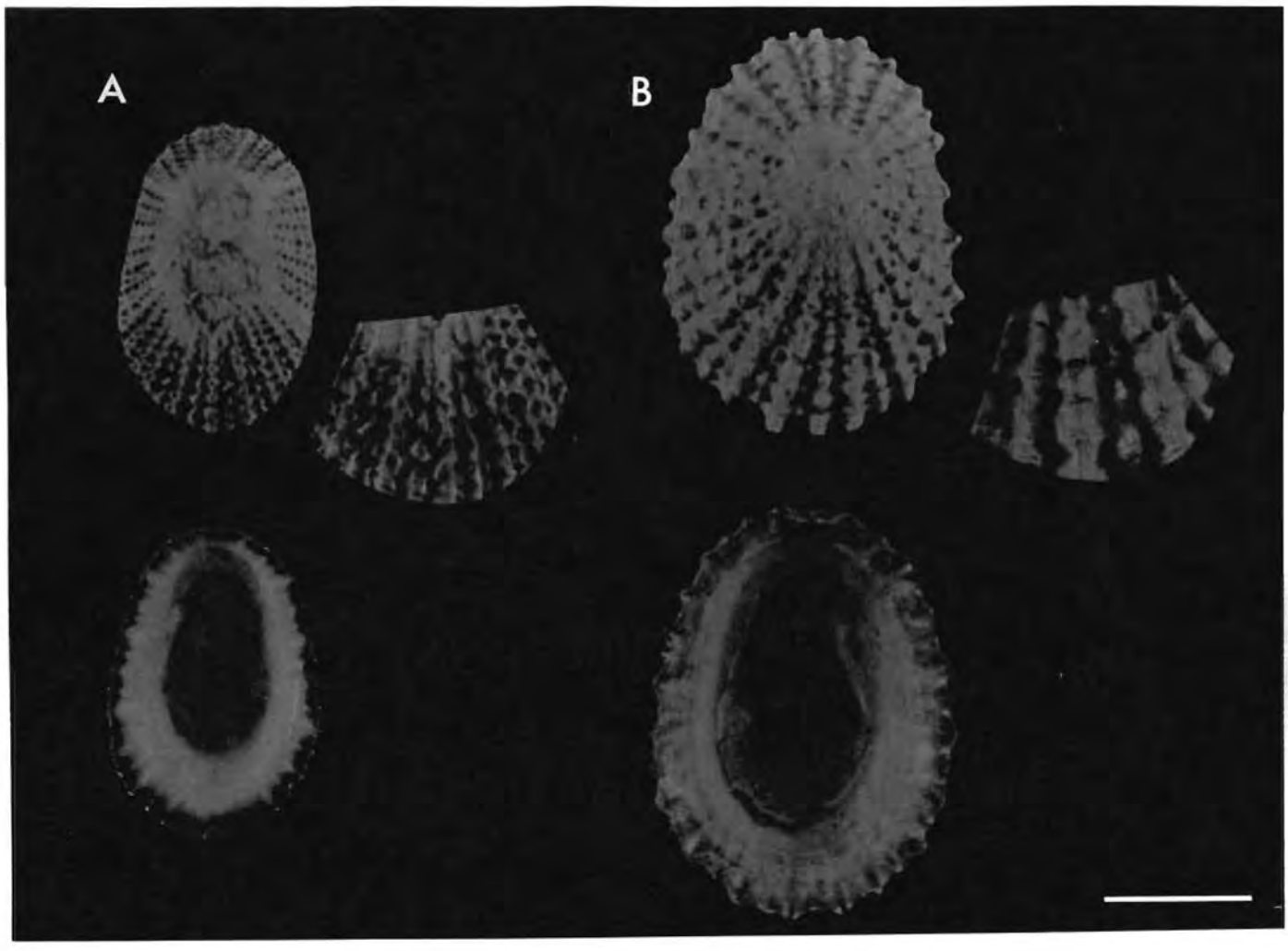


Figure 3.1 Dorsal and ventral views of the shell of (a) *Patella natalensis* and (b) *P. granularis*. Scale bar: 12.5mm.

Sperm - Very elongate, tapering, 'flask-shaped' nucleus, capped by a cone-shaped acrosome with a long anterior extension. Sperm is classified as Type II sperm (Hodgson *et al.*, 1996).

Etymology: Krauss (1848) named this species after the region in which it was first discovered, namely Natal (now known as KwaZulu-Natal) on the east coast of South Africa.

Material examined: South African east coast: SAM A54160, 12 dry shells, sexes unknown, Coffee Bay, collected March 1997 by T. Ridgway. SAM A53165, five dry shells, sexes unknown, Port Edward, collected 16 May 1939 by the University of Cape Town Ecological Survey. SAM A54161, 15 dry shells and 10 whole animals, sexes unknown, Oslo Beach, collected August 1996 by T. Ridgway. SAM A54162, 79 dry shells and 10 whole animals, sexes unknown, Green Point, collected June 1996 by K. Sink. SAM A54163, 32 dry shells and 10 whole animals, sexes unknown, Mapelane, collected August 1996 by G. Branch.

Distribution: So far, found from Coffee Bay (31°2'S 29°8'E) in the Transkei to Mapelane (28°24'S 32°22'E) in northern KwaZulu-Natal. However, its distribution probably extends further northwards than Mapelane on the KwaZulu-Natal coast, possibly extending as far as southern Mozambique. A shell illustrated by Kalk (1995) from Inhaca Island, Mozambique, is likely to be this species, but subsequent searches at Inhaca have failed to detect it (G. Branch pers. comm.). The southern range probably does not extend much further south, because at Dwesa (32°18'S 28°18'E) in the Transkei, *P. natalensis* is absent and replaced by *P. granularis*.

Habitat: This species is one of the most common limpets on the shore in central KwaZulu-Natal. It occurs on the rocks in the mid- to high-shore zone of the rocky shore, often found in association with barnacles (*Tetraclita serrata, Octomeris angulosus* and *Chthamalus dentatus*) or on the brown mussel, *Perna perna*. It occurs higher on the rocky shore than any of the other patellid species within its range.

Comparison with P. granularis: (Figs 3.1 - 3.7; Tables 3.1 - 3.3) *Patella natalensis* is very similar to *P. granularis* but, nevertheless, based on external appearances the two are easily distinguishable (Table 3.1). The nodules on the costae of *P. natalensis* are black, whereas *P. granularis* has white nodules (Fig. 3.1). The number of nodules on the costae differ significantly (ANOVA, $p<0.05$) between the two species for both the anterior and posterior costae. The mean number of costae on the shells also differs significantly (ANOVA, $p<0.05$) between *P. natalensis* and *P. granularis*, although the ranges do overlap.

Morphometrically, the discriminant functions analysis (Fig 3.2) showed that despite the considerable overlap between *P. natalensis* and *P. granularis*, some differentiation is evident, with the trend for the first canonical variable reflecting the fact that the shells of *P. natalensis* are smaller than those of *P. granularis* (Table 3.2). The classification function indicated that, based on SL, SW, SH, WT and VOL, 86.5% of the shells could be correctly identified. However, of the 143 *P. natalensis* shells examined, only 60.2% were correctly reassigned to this species, with the remaining 39.8% being placed within the *P. granularis* grouping. On the other hand, of the 458 *P. granularis* shells examined, 94.8% were correctly reassigned to *P. granularis*, with only 24 shells (5.2%) being placed in the *P. natalensis* group.

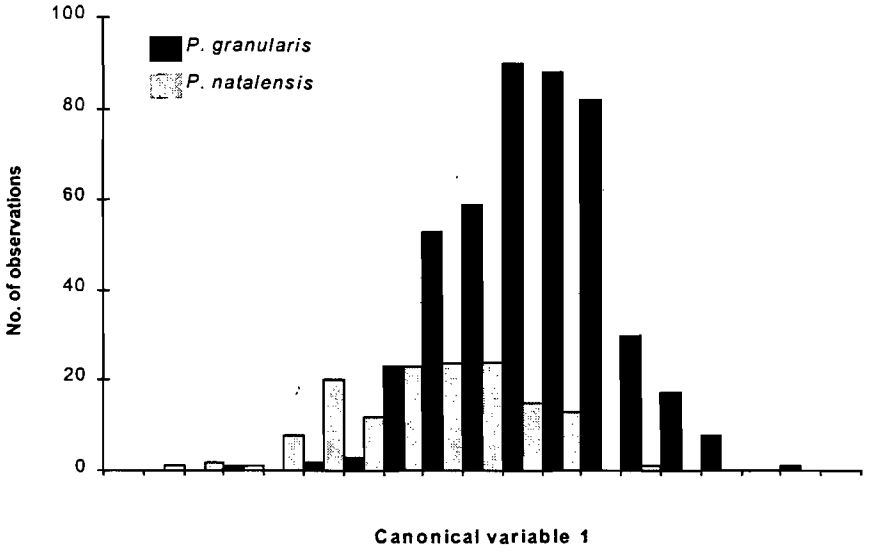


Figure 3.2 Histogram showing the first canonical variable and the number of observations resulting from the discriminant functions analysis for *Patella natalensis* and *P. granularis*.

Table 3.1. Qualitative factors and measurements (Mean±SD) of *Patella natalensis* and *P. granularis*.

	P. natalensis	*P. granularis*
Nodule colour	black	white
No. of costae	43.2 ± 2.44	44.3 ± 2.37
No. of nodules per costae :		
anterior	6.28 ± 0.75	5.73 ± 0.74
posterior	6.64 ± 0.94	6.05 ± 0.82
Foot colour :		
underside	light grey	creamy-orange to creamy-grey
Foot colour :		
side	grey	creamy with grey flecks, or grey
Head colour	grey	cream

Table 3.2 Shell characteristics of *Patella natalensis* and *P. granularis*.

	P. natalensis		*P. granularis*	
	Mean ± SD	Range	Mean ± SD	Range
Shell length (mm)	21.3 ± 5.13	11.68 - 32.64	31.2 ± 7.92	13.92 - 66.49
Shell width (mm)	15.4 ± 3.88	7.50 - 22.89	23.4 ± 6.94	10.04 - 54.07
Shell height (mm)	8.5 ± 2.24	4.03 - 16.11	11.3 ± 4.35	4.78 - 34.97
Shell volume (ml)	0.8 ± 0.53	0.11 - 3.26	2.7 ± 3.00	0.21 - 23.04
Shell weight (g)	0.8 ± 0.61	0.10 - 2.54	2.4 ± 2.41	0.25 - 19.40

The relationships between the shell width and shell length, and shell height and shell length of both *P. natalensis* and *P. granularis* are shown in Figs. 3.3 - 3.4. Generally, shells of *P. natalensis* are more narrow than those of *P. granularis* but they are significantly taller than *P. granularis* shells. The slopes of the relationships in Figs 3.3 - 3.4 differ significantly ($p<0.001$). These figures also indicate that the shells of *P. natalensis* are generally smaller than those of *P. granularis*.

The foot coloration of *P. natalensis* ranges from light to dark grey, and therefore differs from *P. granularis*, which has a more cream to orange coloration although it is sometimes flecked with grey (Table 3.1). The head of *P. natalensis* also has a darker coloration than the creamy-coloured head of *P. granularis* (Table 3.1).

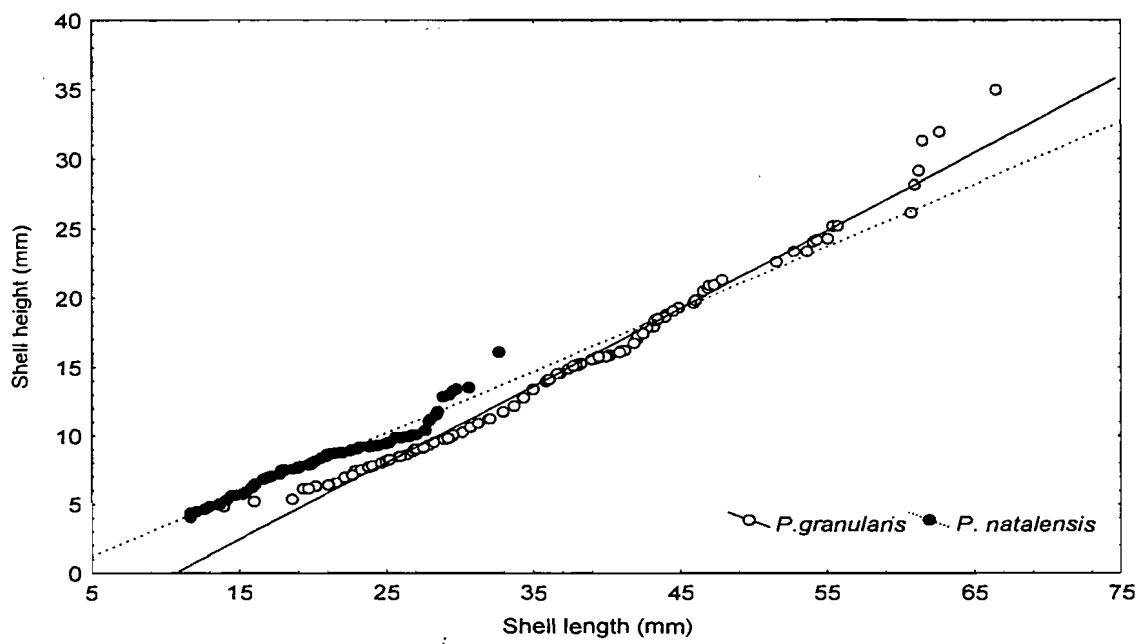


Figure 3.3 The relationship between shell length (mm) and shell width (mm) for *Patella natalensis* (n = 143; y = 0.73*x* - 0.15) and *P. granularis* (n = 458; y = 0.85*x* - 3.15). The two slopes are significantly different ($p<0.001$). All points were included in the analysis, but 351 points (277 O, 74 ●) have been omitted from the figure to prevent obscuring the diagram.

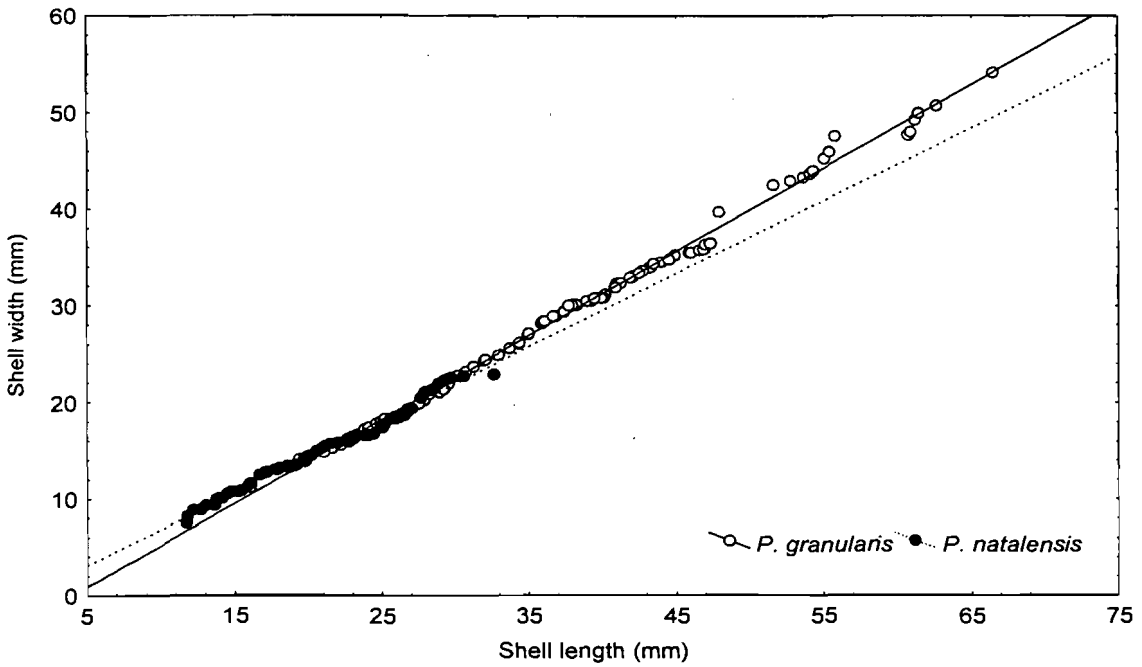


Figure 3.4 The relationship between shell length (mm) and shell height (mm) for *Patella natalensis* (n = 143; y = 0.36*x* + 0.70) and *P. granularis* (n = 458; y = 0.45*x* - 2.76). The two slopes are significantly different ($p<0.001$). All points were included in the analysis, but 351 points (277 O, 74 ●) have been omitted from the figure to prevent obscuring the diagram.

In both *P. natalensis* and *P. granularis*, the X loop of the gut coils neither clockwise nor anticlockwise, and the Y loop is less than 30% of the length of the X loop. However, the length of the Z loop in relation to the visceral mass of *P. natalensis* is significantly shorter (Kruskal-Wallis, $p<0.05$) than that found in *P. granularis* and there is no overlap in this ratio between these two species (Fig 3.5). Thus, the loopings of the mid and hindgut of *P. natalensis* are very similar to those of *P. granularis*, with the exception of the Z loop.

The radulae of both *P. natalensis* and *P. granularis* (Fig 3.6) are typical docoglossate described by the formula 3 + 1 + (2 + 1 + 2) + 1 + 3, and there are no quantitative or structural differences between the radulae of the two species.

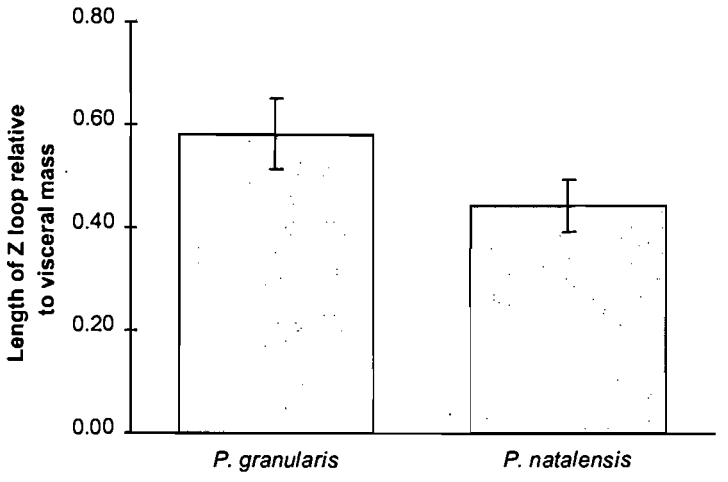


Figure 3.5 Histogram (Mean±SD) of the length of the Z loop relative to the visceral mass of *Patella natalensis* and *P. granularis*..

The sperm of both *P. natalensis* and *P. granularis* is classified as Type II sperm (Hodgson *et al.*, 1996), having elongate tapering 'flask-shaped' nuclei (Fig 3.7). The nucleus is capped by a cone-shaped acrosome with a long anterior extension. The sperm head and mid-piece length and the length of the nucleus show no significant differences between the two species (Table 3.3). However, the lengths of the acrosome and the subacrosomal space are significantly shorter (ANOVA, $p<0.05$) in *P. natalensis* when compared with *P. granularis* (Table 3.3).

Table 3.3 Sperm dimensions of *Patella natalensis* and *P. granularis* (μm).

	P. natalensis	*P. granularis*
	Mean ± SD	Mean ± SD
Sperm head and mid-piece length	13.52 ± 0.57	14.08 ± 0.96
Nucleus length	9.48 ± 0.67	9.52 ± 0.46
Acrosome length	2.05 ± 0.08	2.54 ± 0.15
Subacrosomal space length	1.05 ± 0.06	1.27 ± 0.09

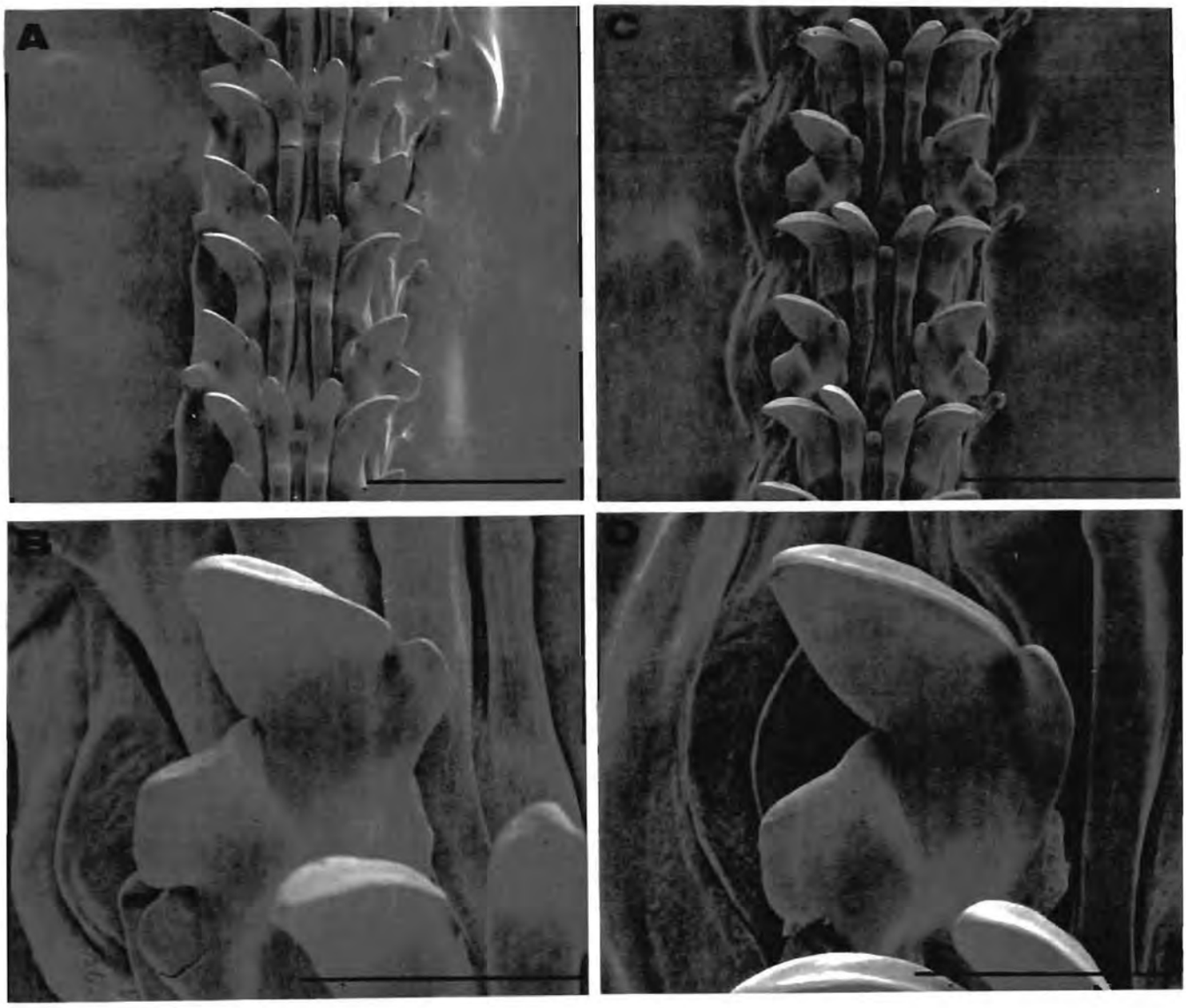


Figure 3.6 *Patella natalensis* (a, b) and *P. granularis* (c, d). Scanning electron micrograph of the radular morphology. (a), (c) overall radular structure. Scale bar: 200μm.
(b), (d) detail of pluricuspid tooth. Scale bar: 100μm.

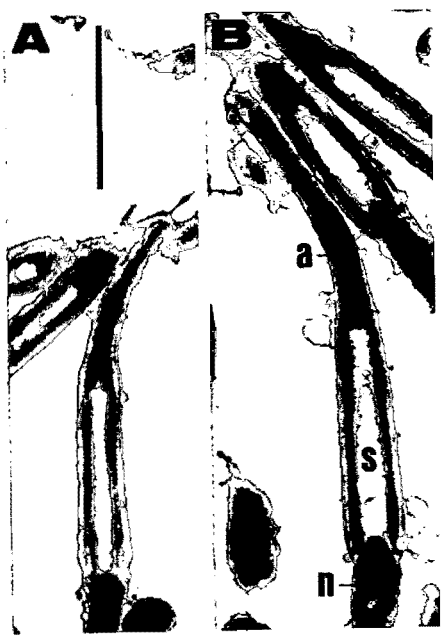


Figure 3.7 Mid-longitudinal section through the spermatozoa of (a) *Patella natalensis*, and (b) *P. granularis*. a, acrosome; s, subacrosomal space; n, nucleus. Scale bar: 1μm.

Comparison with P. miliaris: *Patella natalensis* bears some resemblance to *P. miliaris* from Angola, in so far as that they both have pronounced black nodules on their shells. On the basis of both morphology and mtDNA data, *P. miliaris* is thought to be closely related to *P. granularis* (S. Ridgway pers. comm.) and in a recent phylogeny of the family Patellidae, *P. granularis* and *P. miliaris* form a separate clade (Ridgway *et al.*, in press). *Patella natalensis* is clearly very similar to *P. granularis* (Chapter 2) and can therefore also be assumed to be closely related to *P. miliaris*. However, even though *P. miliaris* has been misidentified as *P. natalensis* in the past (Nicklès, 1950), the two are easily distinguishable. Although both species have black nodules on their shells, the nodules are much darker and more pronounced in *P. miliaris*. The shells of *P. miliaris* also lack the radiating white flashes from the apex of the shell that are evident on *P. natalensis* shells. Furthermore, the external coloration of the *P. miliaris* shells is grey, whereas that of *P. natalensis* is light brown. The internal coloration of *P. miliaris* is consistently white without any radiations as in *P. natalensis*. Also, the colour of the spatula in *P. miliaris* appears to be consistently light brown, whereas in *P. natalensis* the spatula ranges from bluish-white to a dark brown.

Thus, *P. natalensis*, with its pronounced black nodules, is an easily recognisable limpet on the shore which cannot be confused with any other of the southern African limpet species, despite its close resemblance to, and previous confusion with *P. granularis*.

Chapter 4

Limited population differentiation in the bearded limpet *Patella barbara* (Gastropoda: Patellidae) along the coast of South Africa

INTRODUCTION

Southern Africa is of particular interest when dealing with the world-wide distribution of patellid limpets, since about half of the known species are restricted to this region (Ridgway *et al.*, in press), resulting in this area having the highest world-wide diversity of this group. As a consequence of this diversity, coupled with the important influences that limpets have on community structure, much is known about the biology and behaviour of southern African limpets (see Branch, 1981). Despite this, there are two obvious gaps in knowledge which urgently need to be addressed: larval biology; and genetic structure and gene flow.

Patella barbara Linnaeus, 1758, is one of the most widely-distributed of all the southern African limpet species, and inhabits rocks coated with encrusting corallines in the infratidal to the lower Balanoid zone of the shore (Branch, 1971; Kilburn and Rippey, 1982). According to Koch (1949) and Powell (1973), *P. barbara* occurs along the entire coastline from Port Nolloth on the west coast of South Africa through to Umpangazi on the east coast, becoming rare on the east coast of KwaZulu-Natal. Throughout this range *P. barbara* shows variation in shell size, colour and morphology (Stephenson, 1937; Koch, 1949; Branch, 1971). Shells from the west coast are strong, with few costae, whereas shells from False Bay and the Cape Peninsula have numerous costae (Koch, 1949). Shells from the east coast are much flatter than those from further west and, like the west-coast shells, have few costae.

In addition to differences in shell morphology, *P. barbara* also exhibits behavioural differences along the South African coast. According to Branch *et al.* (1992), individuals from the south and east coasts of South Africa show territoriality in that they 'garden' algal resources, whereas those from the west coast do not exhibit this behaviour. 'Gardening' can be defined as the 'modification of plant assemblages, caused by the activities of an

individual grazer within a fixed site, which selectively enhances particular plant species and increases the food value of the plants for the grazer' (Branch *et al.*, 1992). Thus, there appears to be a difference in behaviour from individuals from the east and west coasts.

According to Stephenson (1937), Koch (1949) and Powell (1973), *P. barbara* is the most variable of all the South African limpet species, thus giving rise to confusion if classification of this species is attempted on shell characters alone. This variability has led to a fair amount of taxonomic uncertainty in the past, and *P. barbara* is thus graced with a rather lengthy synonymy (see Powell, 1973). Given the variations in shell morphology and behaviour between west and east coast individuals of *P. barbara*, it was decided to investigate whether these differences were mirrored by other morphological and/or genetic differences. Although similar studies have been carried out on numerous other marine molluscs (Heller and Dempster, 1991; Boulding, Buckland-Nicks and Van Alstyne, 1993; Sanjuan, Pérez-Losada and Rolán, 1997), very few published studies integrate morphological and genetic characters of patellid limpets. Furthermore, such work on patellid limpets is largely restricted to the northern hemisphere (Cretella *et al.*, 1994; Côrte-Real *et al.*, 1996a,b) with Chapter 2 being the first study to integrate both morphological and genetic techniques to assess the taxonomic status of a southern African patellid limpet.

Thus, this chapter is only the second study to investigate genetic differentiation between populations of a southern African patellid limpet species, and therefore provides an insight into gene flow along the coast, as well as determining if genetic differentiation occurs between populations with morphological or behavioural differences. Genetic differentiation was examined by investigating allozyme variation, as determined by protein gel electrophoresis. The use of allozyme variation, although not providing an exact measure of the variation in the encoding DNA, does provide a reliable relative measure. In addition, shell morphometrics, radular morphology, gut-loop coiling and sperm microstructure were examined to test whether the differences in shell morphology and behaviour between the west and east coast populations of *P. barbara* were mirrored by other morphological and/or genetic differences.

MATERIALS AND METHODS

Collection

Specimens of *Patella barbara* were collected from the low intertidal and shallow subtidal zones from Groen River (Groen), Kommetjie (Kom), Clovelly (Clov), Arniston (Arn), Mossel Bay (MBay), Knysna (Kny), St Francis Bay (StFran), and Dwesa (Dwesa) (Fig. 4.1). Foot and mantle tissues were dissected from 10 to 40 individuals from each site, placed in nunc cryopreservation tubes and stored in liquid nitrogen. Fifteen animals from each site, together with the remains of the dissected animals were preserved in 70% alcohol. Upon returning to the laboratory, the dissected tissue samples were subsequently transferred from the liquid nitrogen to an ultra deep-freeze where they were stored (at -80°C) until required for genetic analysis.

Morphological analyses

Shell texture and soft-part coloration: The number of costae on the shells was counted for 10 individuals from each of the sample sites, with the exception of Clovelly where 20 individuals were counted. Differences in costae number 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 pigmentation of the head, tentacles, mouth, underside and the side of the foot was recorded from all the sample sites.

Shell morphometrics: Quantitative measurements of 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) of the shells of 168 individuals from the eight sample sites were measured to the nearest 0.05 mm using vernier callipers. In addition, shell dry weight (WT) was measured and the internal shell volume determined from the weight of 70% alcohol which filled an upturned shell. Morphological variation between the eight populations was analysed by stepwise discriminant functions analysis. The five

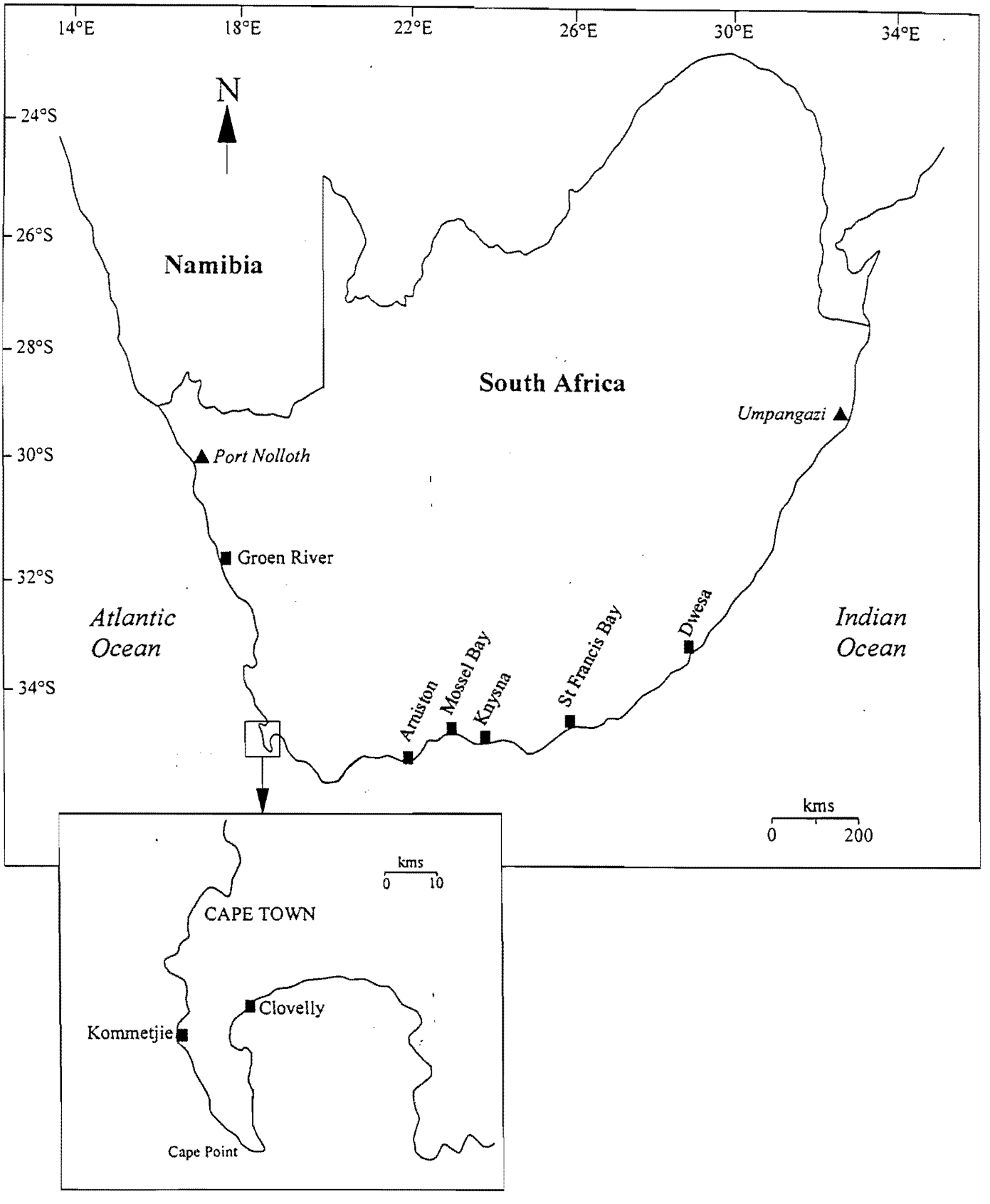


Figure 4.1 Map of South Africa showing the eight sites (■) at which *Patella barbara* was sampled, and the sites mentioned in the text (▲).

morphometric measurements (SL, SW, SH, WT and VOL) taken from each individual were log transformed and analysed as in Chapter 2.

Radular morphology: The radulae of specimens from each of the eight sample sites were examined using a Cambridge S-200 Scanning Electron Microscope (see Chapter 2 for details).

Gut looping: Ten individuals from each of the eight sample sites were dissected and the patterns of the loops of the mid and hindgut examined following Chapter 2. Figure 4.2a shows the structure of the gut and the position of the X and Z loops (the Y loop was absent). Four measurements (Fig. 4.2b) were taken from the gut loops of each individual and three ratios calculated: (a) Z2 / Z1 = ratio indicating the length of the Z loop relative to the visceral mass, (b) LenX / Z1 = ratio indicating the length of the 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. Statistical tests follow those outlined in Chapter 2.

Sperm microstructure: Small portions of the testis of five males from each of the sites (except Knysna) were placed in 4% formalin and other portions in 2.5% gluteraldehyde in filtered sea water and processed at the Department of Zoology and Entomology at Rhodes University. The formalin-fixed samples were processed for light microscopy. Twenty replicate measurements were made of the sperm head and mid-piece length for five individuals from each of the seven sample sites. Nested ANOVA was used (Statistica for Windows Release 5.1, StatSoft Inc., 1996) to test for any significant differences between the populations. The gluteraldehyde-fixed samples were processed for transmission electron microscopy (see Chapter 2 for details). Using the Image Measuring System (IMS) of the JEOL 1210 TEM, two measurements were taken from the TEM images from 15 mid-longitudinal sections per individual from the seven sample sites: (a) length of the nucleus, and (b) length of the acrosome. Nested ANOVA and Tukey's honestly significant difference test were used to test for any significant differences between the populations.

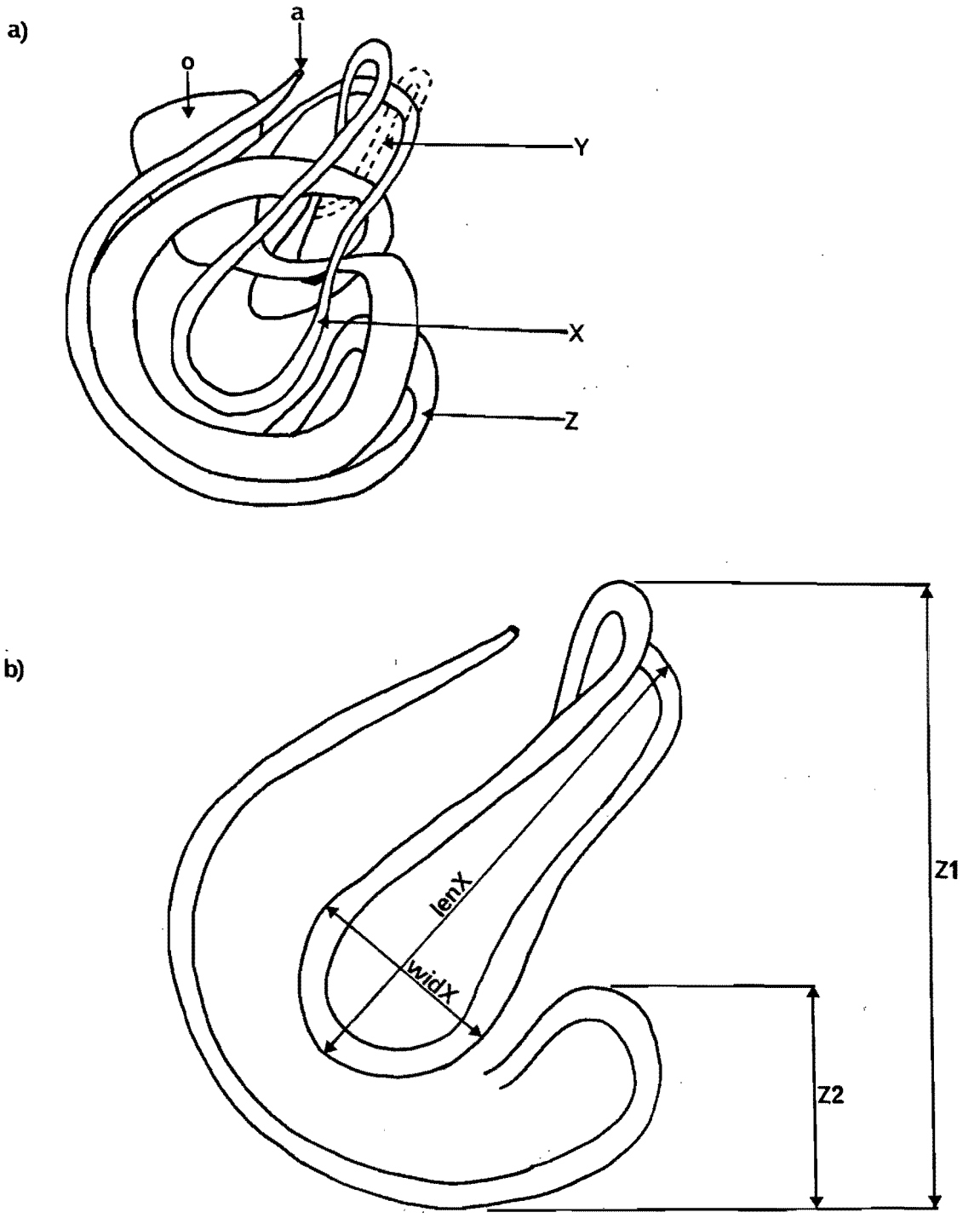


Figure 4.2 Diagrammatic representation of the gut loops of *Patella barbara* after complete dissection from the digestive gland showing (a) the relative positions of the X and Z loops, and (b) the dimensions of the X and Z loops measured. The Y loop is included to show its position if it were present. See Methods for identities of the measurements. a, anus; o, oesophagus.

Electrophoretic analyses

Standard horizontal starch gel electrophoresis (see Harris and Hopkinson, 1976) was carried out to assess the genetic variation at 17 allozyme loci. The loci were chosen without any prior knowledge of their polymorphism. Foot and mantle tissue samples were prepared for electrophoresis following the protocol in Chapter 2. However, an additional buffer system (d) Tris-citrate buffer, pH 8.0 (Ward and Beardmore, 1977) was included. Gels were run for 3.5 to 4.5 hours at a constant current of 40 mA. The enzymes stained for, the buffer systems and the tissues used are listed in Table 4.1.

Numerical analyses were analysed using the programme BIOSYS-1 Release 1.7 (Swofford and Selander, 1981). Allele and genotype frequencies were calculated, and departures from Hardy-Weinberg equilibrium were measured, as in Chapter 2. Average heterozygosity (H) per locus for each population was calculated using Nei's (1978) unbiased estimator. The percentage of polymorphic loci in each population was determined, with a locus being considered polymorphic if the frequency of the most common allele did not exceed 0.99. The F statistics, including $F_{(IS)}$ (the mean value of genetic differentiation or inbreeding coefficient within subgroups), $F_{(IT)}$ (the mean value of genetic differentiation over the entire population), and $F_{(ST)}$ (the genetic differentiation between any two subpopulations) were calculated to determine the degree of genetic differentiation among the subpopulations (Wright, 1978). Other analytical approaches follow those in Chapter 2.

Ecological observations

At Clovelly, *P. barbara* was found not only on the rocks but also on the tests of the ascidian, *Pyura stolonifera*. Analysis of variance was used to test for any significant differences between SL, SW, SH, WT and VOL between the *Pyura* and rock dwelling forms. A scatterplot of SL and SH was plotted to show the differences in the shell morphology of the two forms.

Table 4.1 Enzymes, locus abbreviations, buffer systems and tissue types used. See text and Page 21 for details of buffers.

Enzyme (abbreviation)	E.C. number	Locus	Buffer system	Tissue
Arginine kinase (ARK)	2.7.3.3	ARK-1	(a)	Foot
		ARK-2	(a)	Foot
Glucose-6-phosphate (GPI)	5.3.1.9	GPI-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
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
Isocitrate dehydrogenase (IDH)	1.1.1.42	IDH-1	(c)	Mantle
		IDH-2	(c)	Mantle
Aspartate amino transferase (GOT)	2.6.1.1	GOT-1	(c)	Mantle
Phosphoglucomutase (PGM)	2.7.5.1	PGM-1	(d)	Foot
Aldolase (ALD)	4.1.2.13	ALD-1	(d)	Foot

The presence or absence of 'gardens' was recorded for each population. If 'gardens' were present, slides were taken of 10 of them, and their area calculated from the slides, using the Macintosh programme NIH Image, and related to shell length. Algal samples were also collected from the gardens in an attempt to determine which algal species were associated with the *P. barbara* 'gardens'.

RESULTS

Morphological analyses

Shell texture and soft part coloration: There was little variation in the number of costae between the populations, with the exception of the populations from the Cape Peninsula (i.e. Kommetjie and Clovelly) (Fig. 4.3). These had on average significantly more costae per shell than the other six populations (ANOVA, $p<0.05$). The area inside the myostracum on the underside of the shell ranged from pale brown to white but failed to distinguish the populations, and the area outside the myostracum was consistently white. The soft part morphology remained constant in all eight populations. The head was always black, with the cephalic tentacles and mouth being a creamy-orange colour. The underside of the foot was consistently yellow-orange in colour and the side of the foot creamy-orange with mottled black flecks.

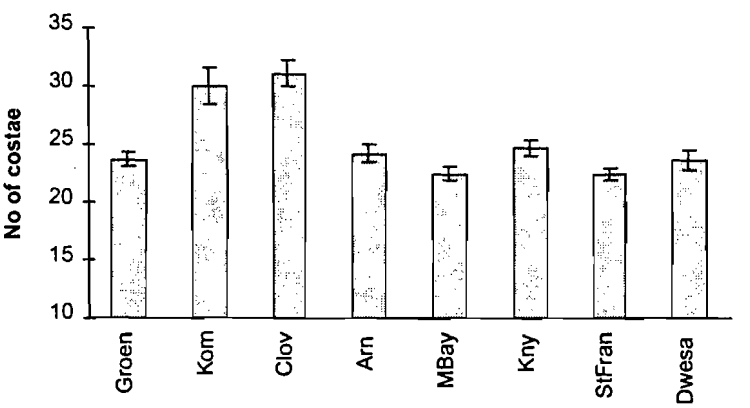


Figure 4.3 Mean (±SE) number of costae per shell for the eight populations of *Patella barbara*.

Shell morphometrics: A summary of the five measurements measured for the eight populations as well as three ratios is shown in Table 4.2. There was no trend in size around the coast with individuals in all populations being of similar mean size. In terms of shell shape (comparing the ratio of SL/SH), it is evident that the shells from the south and east coasts were flatter than those from the west coast. The ratios of SL/SW and WT/VOL displayed no consistent pattern. A discriminant analysis using the five variables was

Table 4.2 Mean and standard error of the shell dimensions measured from 8 populations of *Patella barbara*. See Methods for explanation of abbreviations.

	Population	SL (mm)	SW (mm)	SH (mm)	WT (g)	VOL (ml)	SL/SW	SL/SH	WT/VOL
1	Groen River	58.61±2.41	44.27±1.73	21.79±0.70	13.43±1.29	12.14±0.78	1.32±0.02	2.72±0.12	1.07±0.06
2	Kommetjie	63.15±1.66	50.66±1.14	25.13±1.05	16.46±1.12	14.76±1.97	1.25±0.02	2.57±0.09	1.44±0.23
3	Clovelly	56.25±1.08	46.27±0.86	25.76±0.87	14.92±0.67	14.89±0.67	1.22±0.01	2.29±0.09	1.03±0.03
4	Arniston	68.38±1.58	55.56±1.60	18.38±0.62	19.76±1.39	16.51±1.25	1.24±0.02	3.77±0.11	1.21±0.04
5	Mossel Bay	68.27±3.18	58.68±2.82	19.79±1.29	19.63±2.45	14.55±1.61	1.17±0.02	3.56±0.20	1.36±0.06
6	Knysna	52.76±1.66	41.82±1.81	17.10±0.83	10.92±1.25	8.76±1.10	1.27±0.03	3.14±0.12	1.30±0.10
7	St Francis Bay	61.10±2.13	51.22±2.06	20.22±0.76	15.76±1.99	13.79±1.91	1.20±0.02	3.05±0.09	1.16±0.04
8	Dwesa	58.93±2.83	47.45±2.26	18.93±1.20	13.50±1.82	11.47±1.64	1.25±0.02	3.22±0.15	1.23±0.05

performed on all of the individuals, using populations as the grouping variable. To evaluate population differences, the proportion of specimens in each population that could be correctly assigned to their own population was calculated, and the classification function of the eight populations indicated that only 47.62% of the individuals could be correctly identified. Individuals from none of the populations were consistently assigned to the correct population, with the percentage of correct classifications varying from about 16% for Dwesa to 80% for Arniston. The plot of the first two canonical variables (Fig. 4.4) shows a tremendous amount of overlap between all eight populations of *P. barbara*, with not even any regional differentiation being evident. Most of the separation between populations was along the axis of the first canonical variable, which accounted for about 58% of the total variance. The second canonical variable accounted for only about 23% of the total variability, thus the first two canonical variables account for 81.1% of the variance used to distinguish between the populations. There was a small amount of separation, but this was overwhelmed by major overlaps between all eight populations. Thus, no obvious characteristic of the shell morphometrics differentiated between the populations of *P. barbara*.

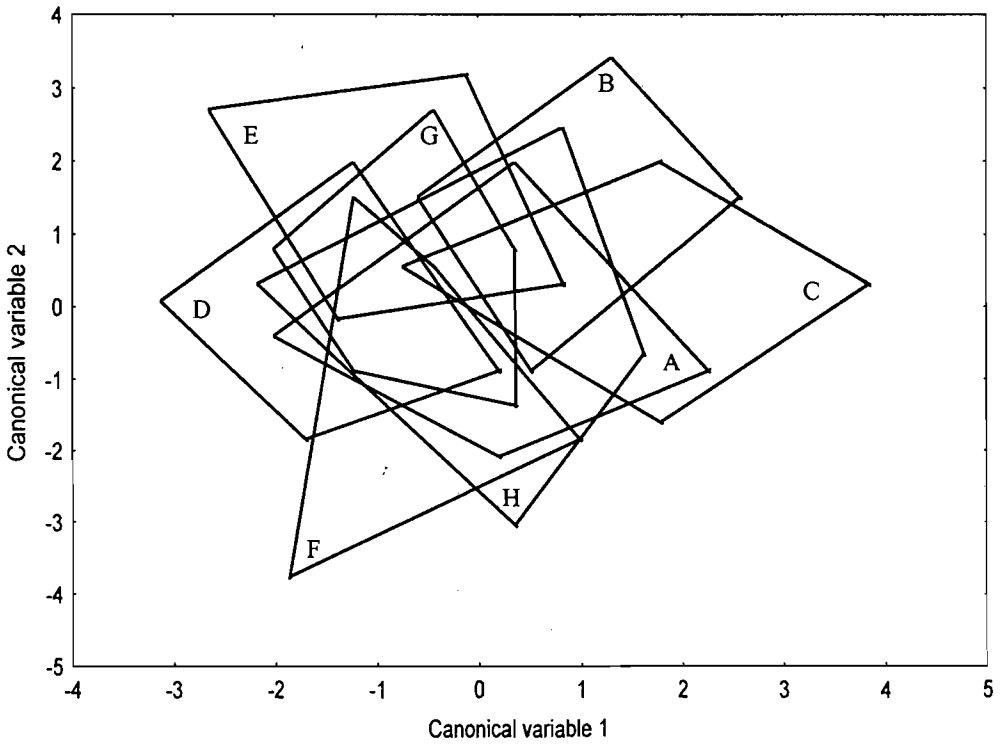


Figure 4.4 Plot of the first two canonical variables for the eight populations of *Patella barbara*. The outlines are convex hulls surrounding Groen River (A), Kommetjie (B), Clovelly (C), Arniston (D), Mossel Bay (E), Knysna (F), St Francis Bay (G), and Dwesa (H). Data points for individuals have been omitted for clarity.

Radular morphology: No difference in radular structure was found between the eight populations. The radula could be described by the formula 3 + 1 + (2 + 1 + 2) + 1 + 3 (Powell, 1973). All radulae had three pairs of unmineralised marginal teeth, three pairs of lateral teeth and a single, large, central rachidian tooth (Fig. 4.5). The lateral teeth are divided into a pair of large pluricuspid teeth and two pairs of smaller unicuspid teeth. The unicuspid teeth form a straight line, with the pluricuspid teeth lying below them, forming a 'stepped' shape. The pluricuspid tooth has four cusps (numbered from the outside to the centre of the radula), with cusp 3 being pointed and larger than cusps 1, 2 and 4.

Gut looping: In all eight populations the X loop of the gut was coiled neither clockwise nor anticlockwise and the Y loop was absent. The means and standard errors of the ratios measured from the X and Z loops for each of the eight populations are shown in Fig. 4.6. The length of the Z loop relative to the visceral mass (Fig. 4.6a) shows that all populations had Z loops of similar length with only Groen River and Arniston differing significantly (Kruskal-Wallis, $p<0.05$) from Knysna. The length of the X loop relative to the visceral mass (Fig. 4.6b) shows that Mossel Bay had a longer X loop than the other populations, but even so, no significant differences (Kruskal-Wallis, $p>0.05$) were recorded. The width relative to the length of the X loop (Fig. 4.6c) showed no significant differences (Kruskal-Wallis, $p>0.05$). Thus, the patterns and loops of the mid and hindgut remained constant throughout the eight populations, or yielded minor differences of no relevance in distinguishing between regions.

Sperm microstructure: Sperm from all eight populations were of similar shape. The spermatozoa have short 'bullet-shaped' nuclei and small undifferentiated acrosomes (Fig. 4.7), and are therefore classified as Type I sperm (Hodgson *et al.*, 1996). Table 4.3 shows the means and standard errors of the three measurements taken from the sperm. Individuals from Groen River had significantly longer sperm heads (ANOVA, $p<0.05$) than those from Kommetjie and Dwesa, whereas individuals from Mossel Bay and Dwesa had significantly smaller nuclei (ANOVA, $p<0.05$) than the other four populations. No significant differences were detected between any of the populations (ANOVA, $p>0.05$) for the length of the acrosome. Thus, although some significant differences were detected, the

Figure 4.5 Scanning electron micrograph of the radular morphology. (a) overall radular structure. Scale bar: 500μm. (b) detail of pluricuspid tooth. Scale bar: 100μm.

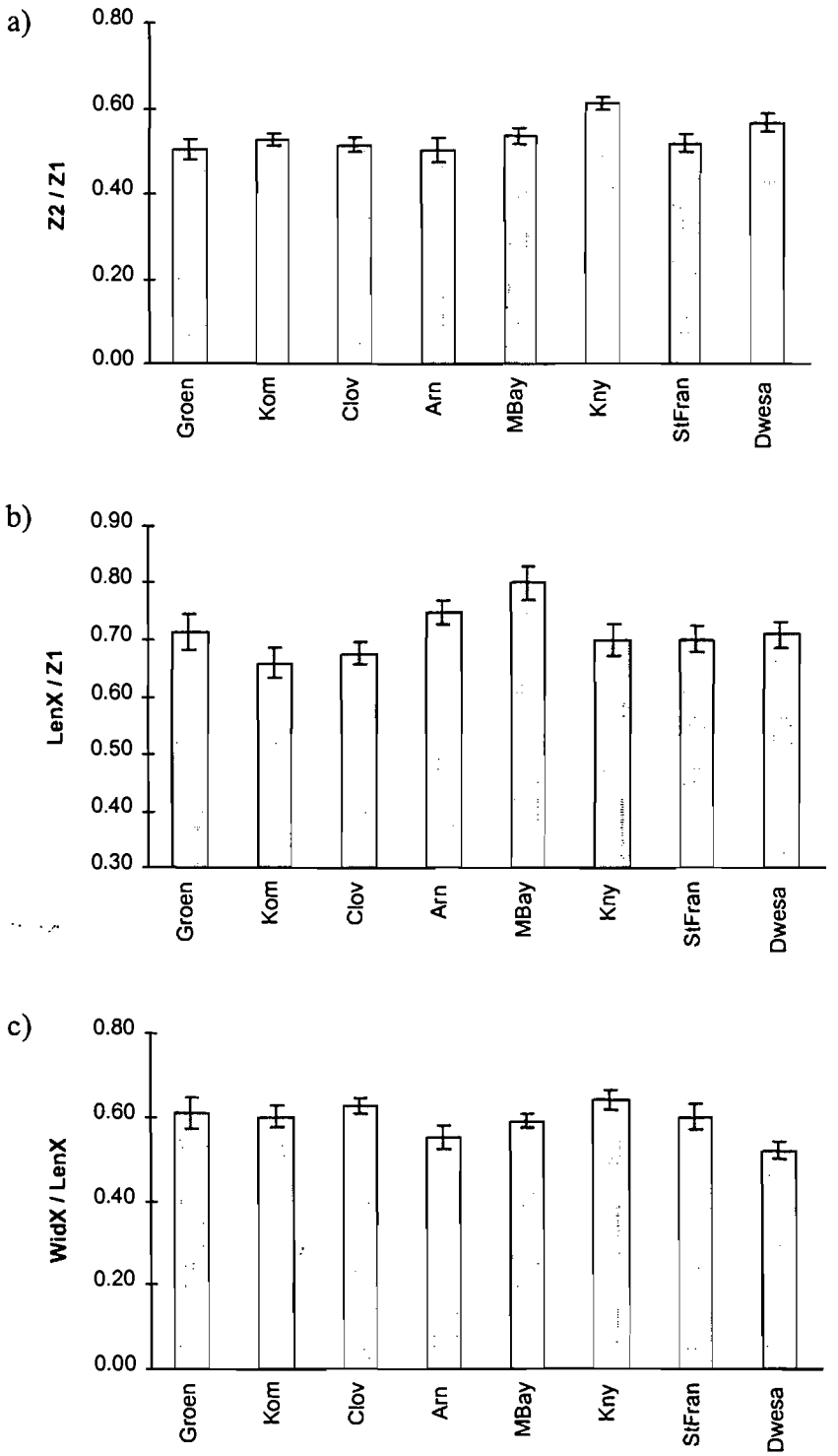


Figure 4.6 Gut loop measurements (mean ± SE) for the eight populations of *Patella barbara*. See Figure 4.2 for interpretation of abbreviations.

sperm microstructural dimensions do not provide sufficient information to be useful in distinguishing between regions.

Table 4.3 Sperm dimensions (mean ± SE) of the eight populations of *Patella barbara*. ND = no data available

Population	Sperm head and mid-piece length (μm)	Nucleus length (μm)	Acrosome length (μm)
1. Groen River	3.53±0.02	2.02±0.01	0.35±0.00
2. Kommetjie	3.46±0.01	2.01±0.02	0.34±0.00
3. Clovelly	3.48±0.01	2.01±0.01	0.34±0.00
4. Arniston	3.48±0.01	2.00±0.01	0.33±0.01
5. Mossel Bay	3.50±0.03	1.95±0.01	0.34±0.00
6. Knysna	ND	ND	ND
7. St Francis Bay	3.48±0.01	ND	ND
8. Dwesa	3.46±0.01	1.96±0.01	0.34±0.00

Electrophoretic analyses

Table 4.4 shows the allele frequencies for the polymorphic loci detected in the eight populations of *P. barbara*. Ten of the loci (ARK-1, SDH-1, SOD-1, ME-1, PHP-1, HEX-1, IDH-1, IDH-2, MDH-1, ALD-1) were consistently monomorphic for all eight populations and have therefore been omitted from Table 4.4. Among the polymorphic loci, the total number of alleles was two in ARK-2, ME-2, GL-2 and GOT-1, and three in GPI-1, PGM-1 and LGG-1. GPI-1 was the only locus that was polymorphic in all populations, whereas at the other extreme, ME-2 and GOT-1 were only polymorphic for Dwesa.

Out of the 35 cases of polymorphism for all loci and populations, nine (25.7%) were found to be out of Hardy-Weinberg equilibrium (χ^2, $p<0.05$). The deviations occurred at five loci, and in all cases occurred due to a deficit of heterozygotes.

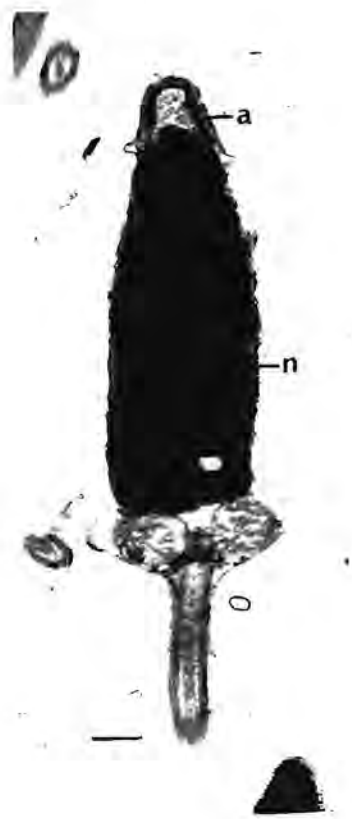


Figure 4.7 Mid-longitudinal TEM section through the spermatozoa of *Patella barbara*. a, acrosome; n, nucleus. Scale bar: 200nm.

Table 4.4 Distribution of allele frequencies at 7 polymorphic loci in 8 populations of *Patella barbara*. (N) = sample size

Locus	Groen River	Kommetjie	Clovelly	Arniston	Mossel Bay	Knysna	St Francis Bay	Dwesa
GPI-1								
(N)	19	25	41	20	13	13	20	19
A	0.921	0.600	0.634	0.900	0.923	0.846	0.800	0.921
B	0.079	0.360	0.317	0.075	0.077	0.154	0.200	-
C	-	0.040	0.049	0.025	-	-	-	0.079
ARK-2								
(N)	19	25	41	16	13	13	20	19
A	0.789	0.780	0.817	0.844	1.000	0.846	0.850	0.816
B	0.211	0.220	0.183	0.156	-	0.154	0.150	0.184
PGM-1								
(N)	19	25	41	20	13	13	20	19
A	0.895	0.900	0.915	0.950	1.000	0.923	0.842	0.842
B	0.053	0.080	0.037	0.025	-	0.077	0.158	0.158
C	0.053	0.020	0.049	0.025	-	-	-	-
ME-2								
(N)	19	25	41	20	13	13	20	19
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.447
B	-	-	-	-	-	-	-	0.553
GL-2								
(N)	19	25	41	20	13	13	20	19
A	1.000	1.000	0.976	0.975	0.731	1.000	0.975	1.000
B	-	-	0.024	0.025	0.269	-	0.025	-
LGG-1								
(N)	19	25	41	20	13	13	20	19
A	0.947	0.740	0.976	0.900	1.000	0.923	0.900	0.947
B	0.026	0.140	-	0.050	-	0.077	0.075	0.053
C	0.026	0.120	0.024	0.050	-	-	0.025	-
GOT-1								
(N)	19	25	41	20	13	13	20	19
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.868
B	-	-	-	-	-	-	-	0.132

The genetic identity (*I*) and distance (*D*) values obtained between the eight populations are shown in Table 4.5. The *I* values obtained for the eight populations ranged from 0.971 - 1.000 (*D* = 0.000 - 0.009). The cluster analysis (Fig. 4.8) revealed a high degree of genetic similarity between the populations. Groen River, Arniston, Knysna and St Francis Bay were genetically identical (*I* = 1.000). Kommetjie and Clovelly were 99.8% similar to each other and separated from the Groen River, Arniston, Knysna and St Francis Bay grouping at an *I* value of 0.996. Mossel Bay separated at an *I* value of 0.992. Dwesa was the most differentiated of the eight populations, largely due to the possession of a second allele in ME-2 and GOT-1, but was still 97.7% similar (*I* = 0.977) to the other populations.

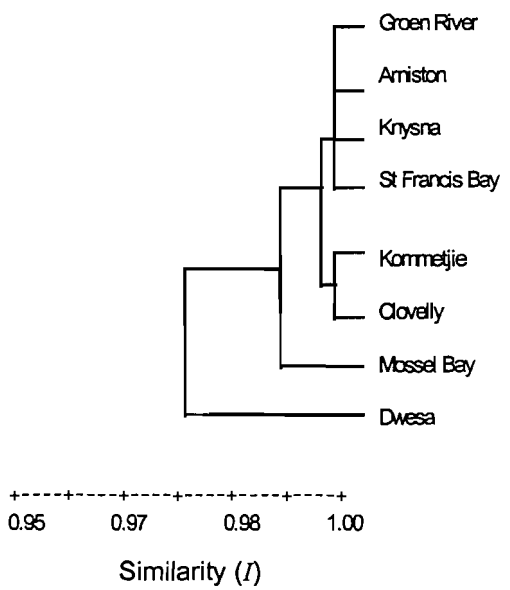


Figure 4.8 UPGMA dendrogram derived from Nei's (1978) genetic identity based on 17 loci for eight populations of *Patella barbara*.

Genetic variation within the populations was examined by calculating average heterozygosities (H), the mean number of alleles per locus, and the percentage of loci which were polymorphic (Table 4.6). The H values varied from 0.033 (Mossel Bay) to 0.093 (Dwesa), and the mean number of alleles from 1.1 (Mossel Bay) to 1.5 (Arniston), whilst between 11.8% (Mossel Bay) and 35.3% (Dwesa) of the loci were polymorphic. The low values encountered for Mossel Bay are probably responsible for its slight differentiation from the other populations. Over the seven polymorphic loci, $F_{(IS)}$ averaged 0.255, whereas the $F_{(IT)}$ averaged 0.347 (Table 4.7). The pairwise $F_{(ST)}$ amongst all of the

populations ranged from 0.031 - 0.519 with a mean of 0.124 (Table 4.7), indicating a small amount of differentiation. However, the mean $F_{(ST)}$ is inflated somewhat by one high $F_{(ST)}$ value of 0.519, for ME-2. Thus, a large proportion of the overall differentiation between the populations was due to ME-2.

Table 4.5 Matrix of Nei's (1978) genetic identity (above diagonal) and genetic distance (below diagonal) averaged over 17 loci for 8 populations of *Patella barbara*.

	Population	1	2	3	4	5	6	7	8
1	Groen River	*****	0.994	0.997	1.000	0.994	1.000	1.000	0.981
2	Kommetjie	0.006	*****	0.998	0.995	0.985	0.997	0.998	0.971
3	Clovelly	0.003	0.002	*****	0.997	0.991	0.999	0.999	0.975
4	Arniston	0.000	0.005	0.003	*****	0.996	1.000	1.000	0.980
5	Mossel Bay	0.006	0.150	0.009	0.004	*****	0.995	0.995	0.973
6	Knysna	0.000	0.003	0.001	0.000	0.005	*****	1.000	0.981
7	St Francis Bay	0.000	0.002	0.001	0.000	0.005	0.000	*****	0.979
8	Dwesa	0.019	0.029	0.026	0.020	0.027	0.020	0.022	*****

Table 4.6 Average heterozygosities (H), the mean number of alleles per locus, and the percentage loci which were polymorphic in the eight populations of *Patella barbara*.

	Population	H (unbiased)	Mean no. of alleles per locus	Percentage polymorphic
1	Groen River	0.047	1.4	23.5
2	Kommetjie	0.087	1.4	23.5
3	Clovelly	0.062	1.4	29.4
4	Arniston	0.047	1.5	29.4
5	Mossel Bay	0.033	1.1	11.8
6	Knysna	0.049	1.2	23.5
7	St Francis Bay	0.052	1.4	29.4
8	Dwesa	0.093	1.4	35.3

Table 4.7 Summary of F-statistics at all the polymorphic loci. See Methods for explanation of abbreviations.

Locus	$F_{(IS)}$	$F_{(IT)}$	$F_{(ST)}$
GPI-1	0.085	0.175	0.098
ARK-2	0.416	0.434	0.031
PGM-1	0.233	0.258	0.033
GL-1	0.672	0.731	0.181
LGG-1	0.289	0.330	0.056
ME-2	-0.171	0.437	0.519
GOT-1	0.309	0.390	0.117
Mean	0.255	0.347	0.124

Ecological observations

At Clovelly, *P. barbara* was found attached to rocks as well as on the tests of the ascidian, *Pyura stolonifera*. The form on *Pyura* had a modified morphology, the shells being significantly shorter and narrower than those occurring on rocks (ANOVA, $p<0.05$, Table 4.8). There was no significant difference between shell weight for the two forms (ANOVA, $p>0.05$, Table 4.8), however, the shells of the *Pyura*-form were significantly taller and had a greater internal volume than the rock-dwelling form (ANOVA, $p<0.05$, Table 4.8). The relationship between the shell length and shell height of the *Pyura*- and the rock-forms are shown in Fig. 4.9. Without exception, shells of the *Pyura*-form were taller and had a greater volume than those of the rock-dwelling form of comparable shell length. The slopes of the relationships in Fig. 4.9 differ significantly ($p<0.001$).

Table 4.8 Mean and standard error of the shell dimensions measured from the *Pyura*- and rock-forms of *Patella barbara* from Clovelly. See Methods for explanation of abbreviations.

	SL (mm)	SW (mm)	SH (mm)	WT (g)	VOL (ml)
Pyura	53.32±0.75	44.31±0.82	28.56±0.91	14.87±0.65	16.06±0.75
Rock	61.00±2.10	49.46±1.56	21.19±0.93	14.99±1.43	12.99±1.16

'Gardens' (as exemplified in Fig. 4.10) were recorded for only two of the eight sample sites, namely Dwesa and Arniston. The 'gardens' varied in size and shape according to the size of the limpet, and contained a scar occupied by the resident limpet, except when it was feeding. The 'gardens' from Dwesa had a mean area of 338 cm^2 (± 43.7) and were larger than those from Arniston, which had a mean area of 60.7 cm^2 (± 7.6). Garden size appears to be related to limpet size, because the size of the gardens increases with an increase in the size of the limpet (Fig. 4.11). The algae associated with these 'gardens' included *Ralfsia verrucosa*, *Ceramium dawsonii*, *Herposiphonia secunda* f. *tenella* and *Herposiphonia* sp. at Dwesa, and *Ralfsia verrucosa* and *Gelidium micropterum* at Arniston.

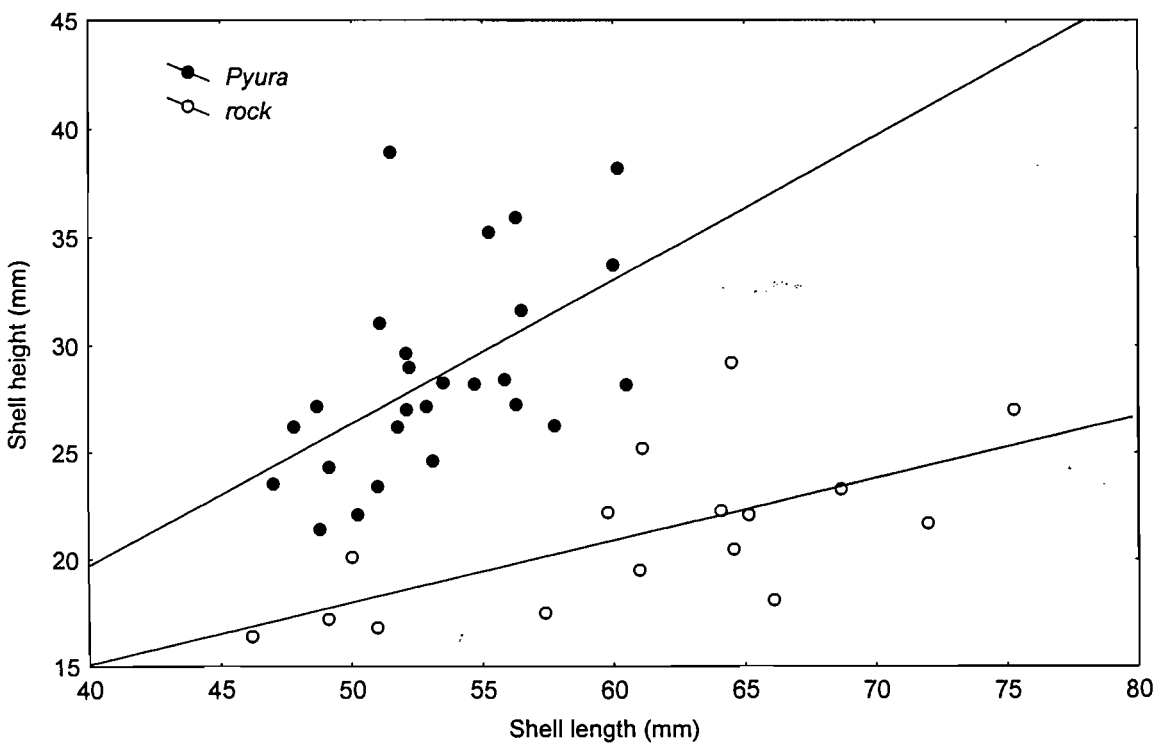


Figure 4.9 The relationship between shell length (mm) and shell height (mm) for *Patella barbara* from the *Pyura* (n = 26; y = 0.67*x* - 6.97) and from the rocks (n = 16; y = 0.29*x* + 3.41). The slopes are significantly different.

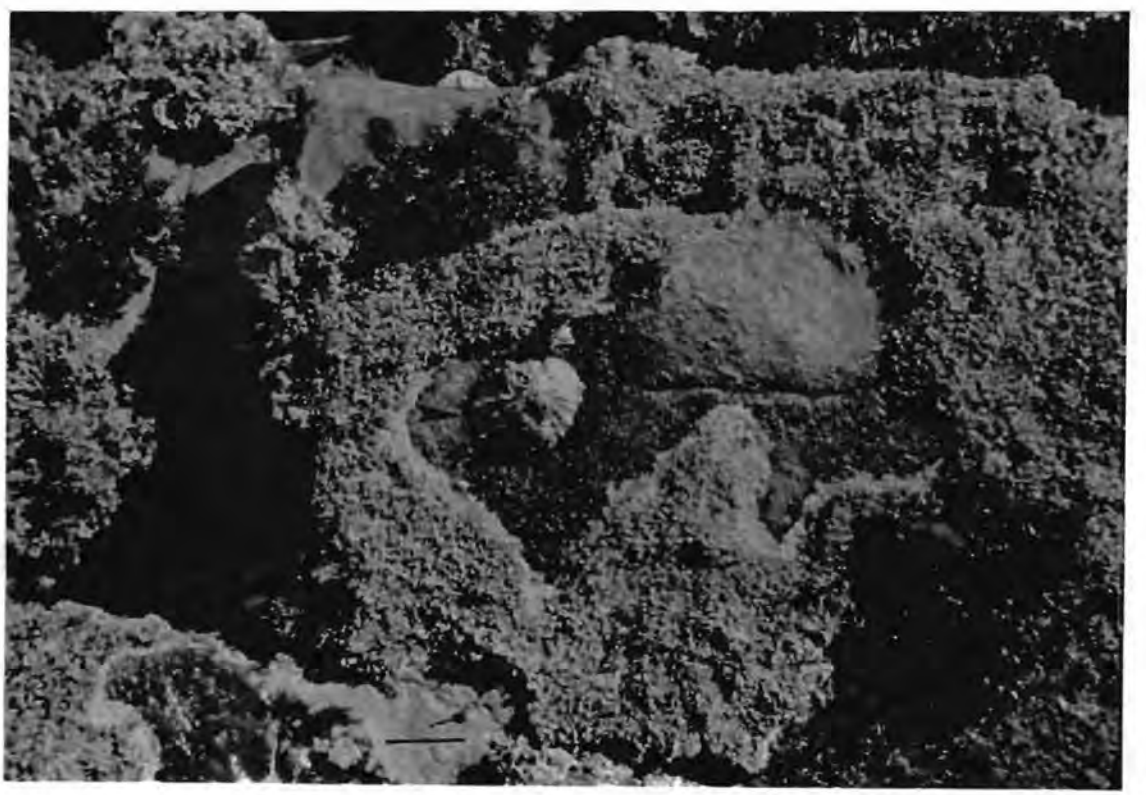


Figure 4.10 Photograph showing an example of a *Patella barbara* 'garden' from Dwesa. Scale bar: 35mm.

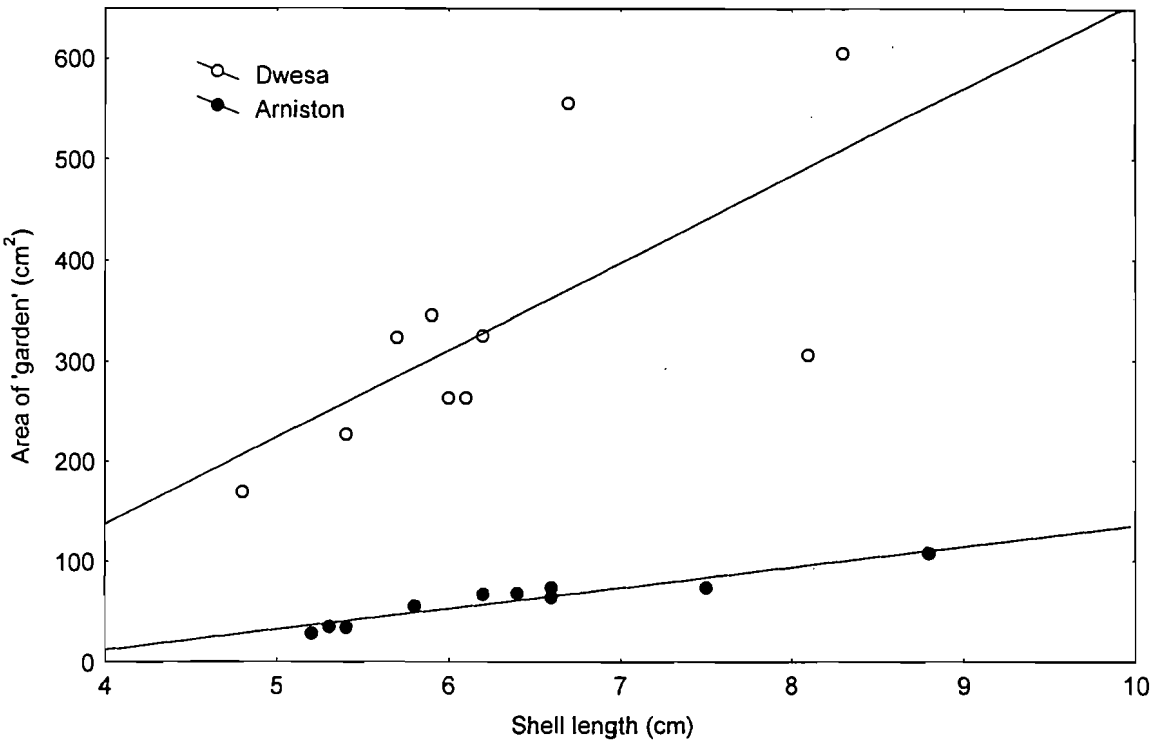


Figure 4.11 The relationship between shell length (cm) and 'garden' area (cm^2) for *Patella barbara* 'gardens' for Dwesa (n = 10; y = 84.84*x* - 198.34) and Arniston (n = 10; y = 20.53*x* -70.30).

DISCUSSION

Differences in shell morphology and behaviour between the west and east coast populations of *Patella barbara* were not mirrored by other morphological and/or genetic differences. The populations of *P. barbara* showed very little morphological or genetic differentiation that could be related to its geographic distribution around the coast of South Africa, and the genetic data suggest that there is very little geographic structuring. The mean genetic identity (Nei, 1978) within this species was 0.995, a value typical of conspecific populations (Thorpe, 1982). If values for other patellid species are taken into account, then the value of 0.995 in this study lies within the range reported for allopatric populations of conspecifics. Côrte-Real *et al.* (1996a) report values of 0.859 for *P. depressa* from Portugal and Spain, 0.898 for *P. caerulea* from Spain and Majorca, and 0.996 for *P. candei* populations from the Azores. The high genetic identity value between the populations of *P. barbara* may be due to the high proportion of monomorphic loci,

which is reflected in the low mean value of polymorphism of 25.7%. This is comparable with values for *P. piperata* (27.3%) and *P. rustica* (33.8%), both of which also contained a high proportion of monomorphic loci (Côrte-Real *et al.*, 1996b).

Marine gastropods exhibit different modes of larval development which potentially influence the extent of gene flow between populations (Grant and Utter, 1988). Electrophoretic studies have shown that species with long-lived planktonic larvae with a large potential for passive dispersal by ocean currents have a large potential for gene flow that leads to genetic homogeneity (Grant and da Silva-Tatley, 1997). Thus, many gastropods with pelagic larvae show genetic homogeneity over large portions of their ranges. However, gastropods with gene flow reduced by larval brooding tend to have genetically-fragmented population structures (Berger, 1973; Janson and Ward, 1984). *Patella barbara* is a broadcast spawner and the genetic data in this study therefore strongly support the expectation of widespread gene flow. This is in contrast to the allegations of Hockey and Branch (1994), who suggest that *Patella* species may be less widely dispersed than would intuitively be expected from marine broadcast spawners, and the data from this study therefore do not support this allegation. The observed $F_{(ST)}$ values were slightly higher than those for other marine species with planktonic larvae (Levinton and Suchanek, 1978; Johnson and Black, 1984). However, the higher mean $F_{(ST)}$ value for *P. barbara* is largely influenced by the high value for ME-2. If ME-2 is excluded, the mean $F_{(ST)}$ value is reduced from 0.124 to 0.086 which is more in line with other studies.

Despite the fact that there appears to be widespread gene flow along the coast of South Africa, some genetic differentiation is evident. The most noticeable differentiation between the populations of *P. barbara* is for the Dwesa population, near the extreme end of the geographic range of this species. This differentiation is largely due to ME-2 and GOT-1, in that the Dwesa population contains a second allele for these two loci which is not present in the other seven populations.

The fact that *P. barbara* has such widespread gene flow along the coast of South Africa has interesting consequences when dealing with speciation. It has been postulated that species with planktonic larvae with the capability for widespread dispersal seem to resist speciation

through the cohesive effect of gene flow (Crisp, 1978; Jablonski, 1986; Grant and Utter, 1988). Such species also tend to have large geographic ranges because they can easily invade favourable habitats. Thus, *P. barbara*, which was shown in this study to be genetically homogenous over approximately 1600 km, provides a good example for this model. Widespread genetic homogeneity has also been shown in Chapter 2 for *P. granularis* over an even greater geographic range, extending from Swakopmund in Namibia to Coffee Bay, a distance of more than 2000 km. However, in contrast to these species with widespread gene flow, species with reduced gene flow and suppressed larval development have greater genetic fragmentation among populations, as shown by Grant and Utter (1988) for *Nucella lamellosa*.

The morphological data largely mirror the genetic data in that no significant differences were detected between the eight populations of *P. barbara*. Although the use of soft-part morphology has been found to be a useful discriminatory tool (Cretella *et al.*, 1994), no differences were found in the colour of the foot, head and tentacles between the populations in this study. Furthermore, comparison of the radulae also revealed no differences, and the patterns of the gut loops remained virtually constant throughout the populations and displayed no geographic trends (Fig. 4.6). Spermatological examinations have also proven useful in distinguishing taxa in numerous studies on patellogastropods (Hodgson and Bernard, 1989; Hodgson *et al.*, 1996; Chapter 2), but the sperm microstructural dimensions examined in this study yielded virtually no differentiation between the populations. However, this result is in itself of interest, because studies on sperm structure seldom make comparisons between multiple sites. Chapter 2 is the only previous study to have done so for patellid limpets. Most spermatological studies on marine molluscs have focused on seeking differences between species, and have examined only one or two sites per species, on the assumption that sperm structure remains constant within species, and that samples from one or a few sites can be considered to be representative of the species. Thus, the results for *P. barbara* and *P. granularis* (Chapter 2) provide novel information on the absence of sperm differentiation within species, even over wide geographic ranges.

Unlike other species of *Patella* along the coast of southern Africa, for which shell length decreases from west to east (Stephenson, 1937; Chapter 2), the shell lengths for *P. barbara*

remained fairly constant along the coast (Table 4.2), attaining the greatest mean length at Arniston on the south coast. The number of costae per shell also showed very little variation between the eight populations, with the exception of the significantly greater number of costae for the Kommetjie and Clovelly populations. This finding is in good agreement with Koch (1949) who reported that *P. barbara* shells from the Cape Peninsula have greater ribbing than shells from elsewhere. Furthermore, the shells from the west coast and the Cape Peninsula had a lower SL/SH ratio, indicating that they are taller than the shells further east. With respect to the morphological characters, the discriminant functions analysis revealed no distinctive groupings (Fig. 4.4) with a great deal of overlap between all eight populations. The chance of correctly re-assigning individuals to their respective populations was as low as 47.6%. The main characters responsible for the discrimination between the populations were shell height and shell width. However, the shape of limpet shells can be modified by a wide range of environmental factors (Bacci and Sella, 1970, Branch, 1981; Lowell, 1984), and the value of these characters in the discrimination is therefore questionable.

At Clovelly, *P. barbara* lives on rocks as well as on the tests of the ascidian, *Pyura stolonifera*. This phenomenon has been reported previously by Koch (1949) and Branch (1971) for *P. barbara* from False Bay and the Atlantic side of the Cape Peninsula. The *Pyura*-forms have a modified morphology, being taller and having a greater internal volume than the rock-dwelling forms of equivalent shell length. This relates to the need for the shells of the *Pyura*-form to fit the curvature of the *Pyura* test. Modification of the shell is thus a phenotypic response to the shape of the substrate, as 'normal' individuals are found on the rocks adjacent to the *Pyura*. Hawkins *et al.* (1990, in Côrte-Real *et al.*, 1996a) report a similar phenomenon for *P. candei* in the Azores, where shell shape is affected by the nature of the substrate, with shells from a softer substratum (where the limpet can excavate a protective scar), being strongly sculptured. On the other hand, shells from harder rock exhibit a smoother shell sculpture. The relatively smaller size of the *Pyura*-form of *P. barbara* is also probably related to the limited amount of food available on the *Pyura* tests when compared with that on the adjacent rocks. Thus, the shell shape and size of *P. barbara* are clearly plastic, even within a single locality.

The presence of algal 'gardens' is not a new phenomenon when dealing with limpets. Defence of algal territories has been demonstrated for the limpets *Lottia gigantea* (Stimson, 1970), *Patella longicosta*, *P. tabularis* and *P. cochlear* (Branch, 1975). However, very little information is available on the 'gardening' activities of *P. barbara* other than the fact that Branch *et al.* (1992) mention that *P. barbara* is an unusual species in that it only gardens over a portion of its distribution. 'Gardens', although possessing low algal species richness, have a high local productivity relative to adjacent areas (Russ, 1987) because continual grazing maintains the algal species in an early, rapid phase of growth. Branch *et al.* (1992) mention that *P. barbara* only 'gardens' on the east coast of South Africa. Since a strong intertidal primary productivity gradient exists around the South African coast (Bustamante *et al.*, 1995), with production being the highest on the west coast and lowest on the east coast, this finding led to the idea that 'gardening' becomes increasingly important in nutrient-poor waters. This theory is largely supported by the large 'gardens' observed for *P. barbara* at Dwesa, on the east coast of South Africa, where productivity is very low. Similarly, well-developed 'gardens' exist at Hluleka, 50 km north of Dwesa (T. Lasiak, pers. comm.), which is another locality with low productivity. The presence of small gardens at Arniston could also be interpreted this way, for productivity on the south coast is significantly lower than that further west, but higher than on the east coast (Bustamante *et al.*, 1995). However, none of the other south-coast populations in this study exhibited 'gardening' behaviour. The absence of 'gardening' at the other south-coast sites, where productivity is relatively low, thus casts doubt on the generality of the idea that this phenomenon is inversely related to productivity.

In sum, the results of this study therefore indicate that the populations of *P. barbara* along the coast of South Africa are relatively homogeneous both morphologically and genetically, despite the behavioural differences exhibited by the Dwesa and Arniston populations. The large amount of morphological overlap between the populations can possibly be attributed to the morphological plasticity of the limpets in response to the environment. The allozyme data failed to reveal significant population differentiation in *P. barbara*, but do provide valuable information on gene flow in southern African patellid limpets, in that the genetic homogeneity indicates widespread gene flow along the coast of South Africa, associated with the possession of a free-swimming larval stage. However,

the use of mtDNA has been demonstrated by Hurst and Skibinski (1995) to be more sensitive than allozymes in detecting population structuring in patellid limpets, and a study of genetic variation using mtDNA may provide an alternative view of population differentiation in *P. barbara*.

Chapter 5

Population differentiation and taxonomic status of the '*Patella miniata*' species complex in southern Africa

INTRODUCTION

The genus *Patella* contains some of the most common intertidal mollusc species on rocky shores. Despite this, consensus has yet to be reached on the number of species within the genus *Patella*, with the two most recent revisions (Christiaens, 1973; Powell, 1973) differing by as many as eight species. Moreover, these two reviews show marked disagreement about classification above the species level. A recent review of the world's patellids argues that the genus *Patella* is polyphyletic, comprising three clades (*Patella*, *Cymbula* and *Scutellastra*) in addition to *Helcion* (Ridgway *et al.*, in press), requiring the re-assignment of patellid species to these four genera. I support this, but have retained the older broader use of the name *Patella sensu lato* here to facilitate comparisons of species known until now under this name.

The problems of limpet taxonomy are largely attributable to the considerable morphological plasticity of individuals (Côrte-Real *et al.*, 1996a), and the fact that early taxonomy was based purely on morphological characters, notably shell form and radular structure. It is thus hardly surprising that the reviews of Christiaens (1973) and Powell (1973), based purely on shell morphology and radular characters, show numerous disagreements. Nevertheless, despite the confusion surrounding the old genus *Patella*, in comparison with other regions, the southern African representatives of the group are fairly well characterised. There remains, however, some taxonomic uncertainty for a suite of closely related species within the *Cymbula* clade in southern Africa. In their their cladistic phylogeny of the family Patellidae, Ridgway *et al.* (in press) recognise *Patella miniata* Born, 1778, *P. compressa* Linnaeus, 1758, and *P. adansonii* Dunker, 1853, as members of a monophyletic southern African clade. *Patella miniata*, although showing marked colour gradations around the southern African coast (Koch, 1949), is currently recognised as a valid species on South African shores, extending from Port Nolloth to KwaZulu-Natal (Powell, 1973). *Patella sanguinans* Reeve, 1854, on the other hand, was recognised by

Sowerby (1892), Bartsch (1915) and Tomlin and Stephenson (1942) as a valid species, but since then it has been dismissed and included as a synonym of *P. miniata* (Koch, 1949; Christiaens, 1973; Powell, 1973). The variation in the sculpturing and the coloration of the shells of *P. sanguinans* was not considered sufficient to separate it from *P. miniata*, because similar variations have been shown to occur in other species of South African limpets (i.e. *P. barbara* Linnaeus, 1758 and *P. longicosta* Lamarck, 1819) (Koch, 1949). However, Kilburn and Rippey (1982) and Branch *et al.* (1994), although not recognising *P. sanguinans* as a full species, note the differences recorded by Tomlin and Stephenson (1942) and divide *P. miniata* into two subspecies, *P. miniata miniata* and *P. miniata sanguinans*, recording the former from Namibia to the Transkei, and the latter from Transkei and KwaZulu-Natal. Furthermore, Ridgway *et al.* (in press) exclude the possibility of *P. sanguinans* as a species, since they consider it identical to *P. miniata* in the morphological characters examined in their cladistic study, differing only in the size and coloration of the shell.

South-coast specimens of *P. miniata* have typical pink rays and low shells, whereas their counterparts on the west coast, including those in Namibia, are taller and tinged blue. These variants have been given various names, including *P. safiana* Lamarck, 1819 (R. Kilburn pers. comm.) and *P. adansonii* (Ridgway *et al.*, in press). I refer to them here as *P.* cf. *miniata*. The status of these west-coast individuals thus also need resolution.

The kelp limpet, *P. compressa*, although usually recognised as a valid species on South African shores with a range extending from Port Nolloth to Danger Point (Powell, 1973), was considered by Pilsbry (1891) to be conspecific with *P. miniata*. Pilsbry (1891) stated that the species develops into the *compressa* form on kelp, and into the *miniata* form on rocks. Pilsbry's (1891) suggestion did not gain general acceptance and it has been dismissed by numerous authors (Koch, 1949; Christiaens, 1973; Powell, 1973). However, Kilburn and Rippey (1982) mention that *P. compressa* may be indistinguishable from *P. miniata* if it settles on a rocky substrate instead of on kelp stipes. Furthermore, Ridgway *et al.* (in press) also suggest that *P. miniata* and *P. compressa* may prove to be ecomorphs of the same species (living on rocks and kelp respectively).

The aim of this chaper is to examine the species boundaries between *P.* cf. *miniata*, *P. miniata miniata*, *P. miniata sanguinans* and *P. compressa*, and thus provide a revision of the taxonomic status of the '*Patella miniata*' 'clade' (Ridgway *et al.*, in press) in southern Africa. A further species, *P. safiana* Lamarck, 1819, from Angola, which according to Ridgway *et al.* (in press) is closely related to *P. miniata miniata* and *P. compressa*, is included in the study to provide a suitable reference population. Both morphological and biochemical techniques are integrated. Protein gel electrophoresis is an extremely useful technique that allows for the identification of separate gene pools and is of considerable value in distinguishing species (see Avise, 1975; Hillis and Moritz, 1990), and the integration of protein gel electrophoresis with the more traditional morphological approaches has helped to elucidate the taxonomic status of a variety of molluscan species (Skibinski *et al.*, 1980; Hedgecock and Okazaki, 1984; Heller and Dempster, 1991; Cretella *et al.*, 1994; Côrte-Real*et al.*, 1996a,b). Thus, in addition to electrophoresis, soft-part morphology, shell morphometrics, radular morphology, gut-loop coiling and sperm microstructure were examined to assess the taxonomic status and relationships of the species in question. *Patella adansonii* is known only from shells, so that an integrated approach could not be applied to it, but the type specimens were examined to resolve its status.

MATERIALS AND METHODS

Collection

Specimens of *Patella miniata miniata* (abbreviated herein as *min*) were collected from intertidal zones at Kommetjie (Kom), Clovelly (Clov), Still Bay (SBay), Knysna (Kny), St Francis Bay (StFran) and Dwesa (Dwesa). Individuals of *P. miniata sanguinans* (*sang*) were sampled from Dwesa (Dwesa) and Ballito (Ballito) on the east coast. Specimens were also collected from Groen River (Groen) and Swakopmund (Swakop) on the west coast of southern Africa. These specimens were originally identified as *P. adansonii* following Ridgway *et al.* (in press), but I reserved judgement on their identity and therefore refer to them as *P.* cf. *miniata* (cf. *min*). A further sample was obtained from the intertidal zone from southern Angola (Angola) and these individuals were identified, after

examination of the type and original description, as *P. safiana*. *Patella compressa* was sampled from the kelp in the subtidal zone at Kommetjie (Kom) and Clovelly (Clov). Figure 5.1 shows the location of the 13 sample populations examined. With the exception of the specimens collected from Kommetjie and Clovelly, foot and mantle tissues were dissected from each individual, placed in nunc cryopreservation tubes and stored in liquid nitrogen. Upon returning to the laboratory, the dissected tissue samples were transferred from the liquid nitrogen to an ultra deep-freeze where they were stored (at -80°C) until required for genetic analysis. The Kommetije and Clovelly samples were transported live to the laboratory where they were stored in the ultra deep-freeze. Fifteen animals from each site, together with the remains of the dissected animals, were preserved in 70% alcohol.

Morphometric analysis

Quantitative measurements of shell length (SL, greatest distance between anterior and posterior end), shell width (SW, greatest distance perpendicular to the anterior-posterior axis), shell height (SH, greatest vertical distance from the apex of the shell to the plane of the aperture) of the shells of 231 individuals from the 13 sample sites were measured to the nearest 0.05 mm using vernier callipers. In addition, shell dry weight (WT) was measured and the internal shell volume determined from the weight of 70% alcohol which filled an upturned shell. The morphometric measurements were analysed by principle component analysis (PCA) (Statistica for Windows Release 5.1, Statsoft Inc., 1996) to test whether there was any visible separation of the 13 populations based on shell morphometrics alone. The PCA was based on log-transformed variables and the significance of the separation of the groups was tested by one-way analysis of variance (ANOVA) of the overall principle-component scores (Statistica for Windows Release 5.1, Statsoft Inc., 1996).

The shell morphometric data from the 13 populations used in the PCA were further analysed by discriminant functions analysis. The data were grouped into the genetic groupings, log transformed and analysed as in Chapters 2 and 4.

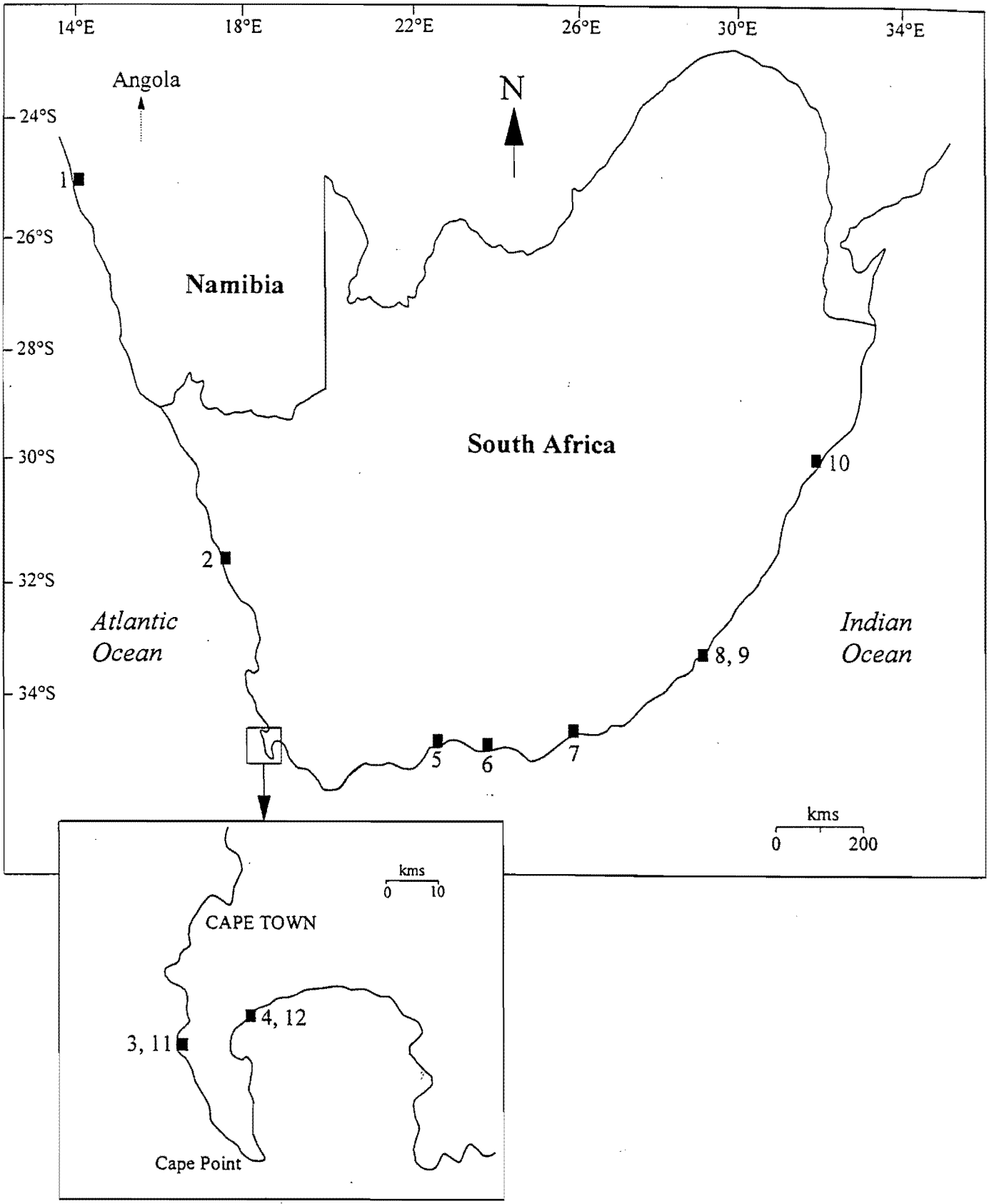


Figure 5.1 Map of southern Africa showing the locations of 12 of the 13 sites (■)at which limpets were sampled. 1. Swakopmund (cf. *min*); 2. Groen River (cf. *min*); 3. Kommetjie (*min*); 4. Clovelly (*min*); 5. Still Bay (*min*); 6. Knysna (*min*); 7. St Francis Bay (*min*); 8. Dwesa (*min*); 9. Dwesa (*sang*); 10. Ballito (*sang*); 11. Kommetjie (*comp*); 12. Clovelly (*comp*). See Methods for species abbreviations.

Electrophoretic analyses

Genetic variation at 16 allozyme loci was examined using standard horizontal starch gel electrophoresis (see Harris and Hopkinson, 1976). The sample sizes used per population are shown in Table 5.4. Foot and mantle tissue samples were prepared for electrophoresis following the protocol in Chapter 2. The enzymes assayed, the buffer systems, and the tissue used are listed in Table 5.1. The electrophoretic data were analysed using the programme BIOSYS-1 Release 1.7 (Swofford and Selander, 1981), following the approaches in Chapters 2 and 4.

Table 5.1 Enzymes, locus abbreviations, buffer systems and tissue types used. See Pages 21 and 66 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
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
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
Isocitrate dehydrogenase (IDH)	1.1.1.42	IDH-1	(c)	Mantle
		IDH-2	(c)	Mantle
Aspartate amino transferase (GOT)	2.6.1.1	GOT-1	(c)	Mantle
Phosphoglucomutase (PGM)	2.7.5.1	PGM-1	(d)	Foot
Aldolase (ALD)	4.1.2.13	ALD-1	(d)	Foot

The taxonomic relationship between the species in question was assessed by a phylogenetic analysis carried out on the allozyme data. Although the cladistic analysis of morphological characters has been widely used for numerous taxa, the cladistic analysis of allozymes for constructing phylogenies is more recent (Buth, 1984; Swofford and Olsen, 1990). Two fundamental problems have arisen with respect to the coding of electrophoretic data: what unit constitutes the character, and how the character states are ordered. Numerous methods have therefore been proposed for the coding of such data (see Buth, 1984; Mabee and Humphries, 1993). One method, the *independent alleles* model (Mickevich and Mitter, 1981), is to treat the allele as the character, with the states being either present or absent. However, this method is not without criticism (Buth, 1984, Swofford and Olsen, 1990) because the alleles at a single locus are not independent, and the approach is biologically unrealistic, since hypothetical ancestors may contain no alleles for some loci. Thus, an alternative approach is to use the locus as the character, with the alleles or allelic combinations as the states. However, this therefore raises the problem of how to order the character states. Mickevich and Mitter (1981, 1983) proposed four methods for inferring character state order, each of which makes different assumptions regarding the theories of allozyme evolution. A further method involves the coding of allele frequency data (for more information see Buth, 1984; Mickevich and Mitter, 1981, 1983).

In this study, two different approaches were employed. Firstly, the *independent allele* model (Mickevich and Mitter, 1981) was used where the allele is the character, and its presence and absence in a taxon are the character states. Secondly, the locus was treated as the character and the allelic combinations at each locus were the character states. These states were ordered using the *minimum allele turnover* model (Mickevich and Mitter, 1983).

A further important consideration in any phylogenetic analysis is the selection of an outgroup. The outgroup comparison method determines the character polarity and according to Watrous and Wheeler (1981), "for a given character with two or more states within a group, the state occurring in related groups is assumed to be the pleisomorphic state". It is commonly held that the most suitable outgroup to choose is that of a closely related species or genus (Ridley, 1986). According to Ridgway *et al.* (in press), *P. safiana*

is closely related to *P. miniata* and *P. compressa*, and it therefore provides a suitable outgroup taxon. Thus, *P. safiana* was used as the outgroup taxon for the analysis in this study.

All the cladistic analyses were performed using the implicit enumeration ('ie') command in the HENNIG86 programme (Farris, 1988). The consistency (CI) and retention (RI) indices were used to measure the fit of the data to the phylogenetic trees. All characters used were unweighted.

Morphological analyses

Shell and soft part morphology: Visual observations were made on the external and internal appearance of the shell, as well as the pigmentation of the head, tentacles, mouth, underside and side of the foot.

Radular morphology: Radulae from each of the populations were examined using a Cambridge S-200 Electron microscope (see Chapter 2 for details).

Gut looping: Individuals from each of the sample populations were dissected and the patterns of the loops of the mid and hindgut examined following Chapter 2. The structure of the gut and the position of the various loops are shown in Fig. 5.2a. Four measurements (Fig. 5.2b) were taken from the gut loops of each individual and three ratios calculated: (a) Z2 / Z1 = ratio indicating the relative length of the Z loop, (b) LenX / Z1 = ratio indicating the length of the 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. Statistical tests follow those outlined in Chapter 2.

Sperm microstructure: Sperm was examined from *P. miniata miniata* from Clovelly and St Francis Bay, from *P. miniata sanguinans* from Dwesa and Ballito, from *P.* cf. *miniata* from Groen River and Swakopmund, and from *P. compressa* from Kommetjie and Clovelly. No sperm samples were available for *P. safiana* from Angola. Small portions of the testis of five males per site were placed in 2.5% gluteraldehyde in filtered sea water and processed

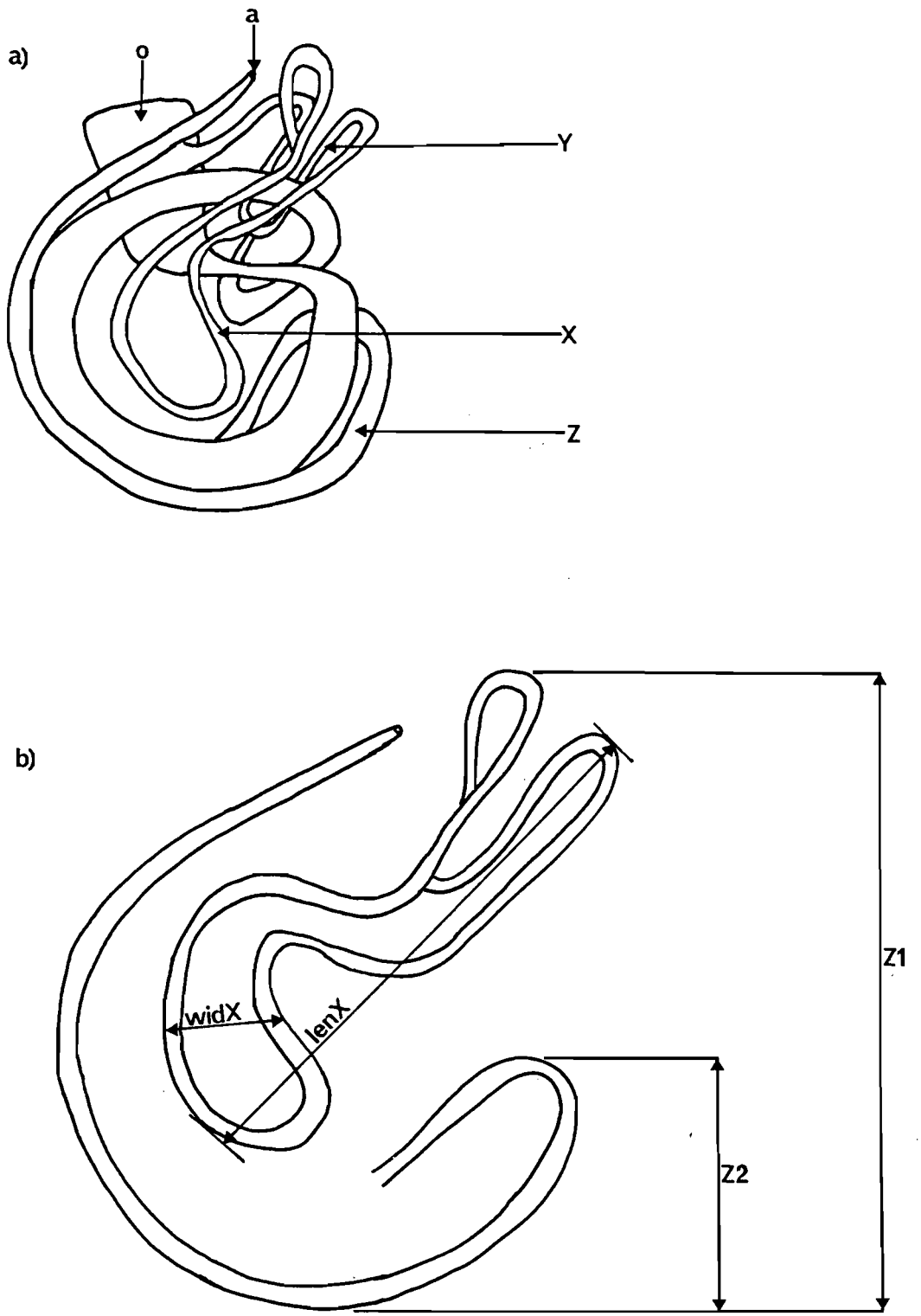


Figure 5.2 Diagrammatic representation of the gut loops after complete dissection from the digestive gland showing (a) the relative positions of the X, Y and Z loops, and (b) the dimensions of the X and Z loops measured. See Methods for identities of the measurements. a, anus; o, oesophagus.

for transmission electron microscopy (TEM) at the Department of Zoology and Entomology at Rhodes University (see Chapter 2 for details). Using the Image Measuring System (IMS) of the JEOL 1210 TEM, the measurements shown in Fig. 5.3 were taken from 10 mid-longitudinal sections per individual per population from the TEM images: (a) total length of the nucleus and acrosome, (b) length of the nucleus, (c) length of the acrosome, and (d) basal width of the acrosome. Differences between the populations were tested using nested ANOVA and Tukey's honestly significant difference test (Statistica for Windows Release 5.1, StatSoft Inc., 1996).

RESULTS

Morphometric analysis

Table 5.2 shows the results obtained from the principle component analysis (PCA). Principle component 1 (PC1) accounted for 81% of the total variation, with the first eigenvector being equally influenced by shell length, shell width, shell height, shell dry weight and shell volume. The equal loading of PC1 suggests that it approximates an isometric size vector. Principle component 2 (PC2) was influenced mainly by shell height, PC3 by shell length, PC4 by shell width and PC5 by shell volume. Principle component 2 accounted for about 14% of the total variation, whereas PC3, PC4 and PC5 collectively accounted for about only 5% of the total variation. The scatterplot of PC2 against PC1 of the populations grouped into species is shown in Fig. 5.4. Overall, groups differ significantly from each other for all five of the principle components (ANOVA, $p = 0.000$). With the exception of the clear separation of the Kommetjie and Clovelly populations of *P. compressa*, the scatterplot revealed little differentiation among the other 11 populations. Therefore, the PCA analysis only clearly distinguished the two populations of *P. compressa* from the other populations.

A discriminant analysis using the five morphometric variables (SL, SW, SH, WT, VOL) was performed on all individuals, using the four genetic groupings as the grouping variable. Figure 5.5 shows a plot of the first two canonical variables for the four genetic groupings. There was a fair amount of overlap between the *P. miniata miniata*, *P. miniata*

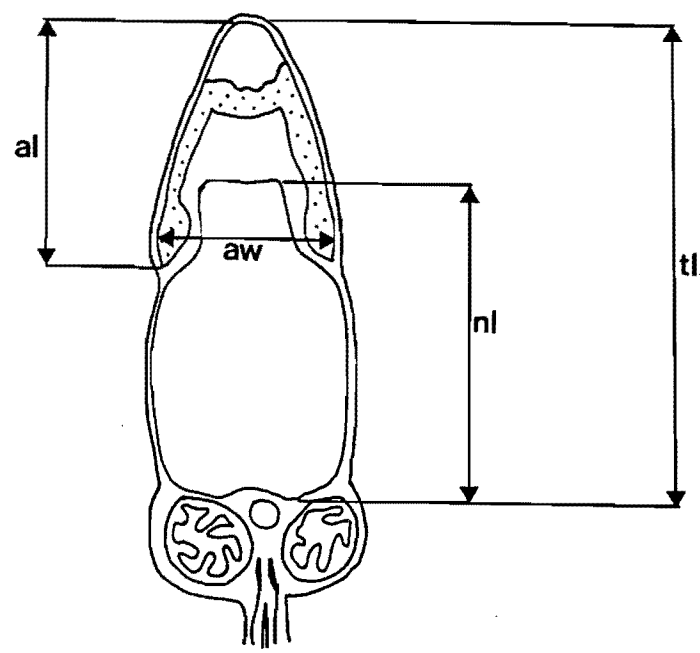


Figure 5.3 Diagrammatic representation of a mid-longitudinal section through a sperm showing the dimensions measured. a, acrosome; al, length of acrosome; aw, basal width of acrosome; n, nucleus; nl, length of nucleus; tl, total length of nucleus and acrosome.

Table 5.2 *Patella* cf. *miniata*, *P. miniata miniata*, *P. miniata sanguinans*, *P. compressa*, and *P. safiana*. Summary of principle component analysis based on five morphometric characters in thirteen populations.

	Principle component				
	1	2	3	4	5
Eigenvalue	4.0528	0.7072	0.1071	0.0795	0.0535
(α of variation)	(0.811)	(0.141)	(0.021)	(0.016)	(0.011)
Eigenvectors					
Shell length	0.9637	-0.0169	-0.2418	0.0327	0.1062
Shell width	0.8224	-0.5379	0.0487	-0.1780	-0.0125
Shell height	0.7659	0.6227	0.0855	-0.1288	0.0398
Shell dry weight	0.9569	-0.1319	0.1894	0.1632	0.0652
Shell volume	0.9720	0.1111	-0.0553	0.0590	-0.1903

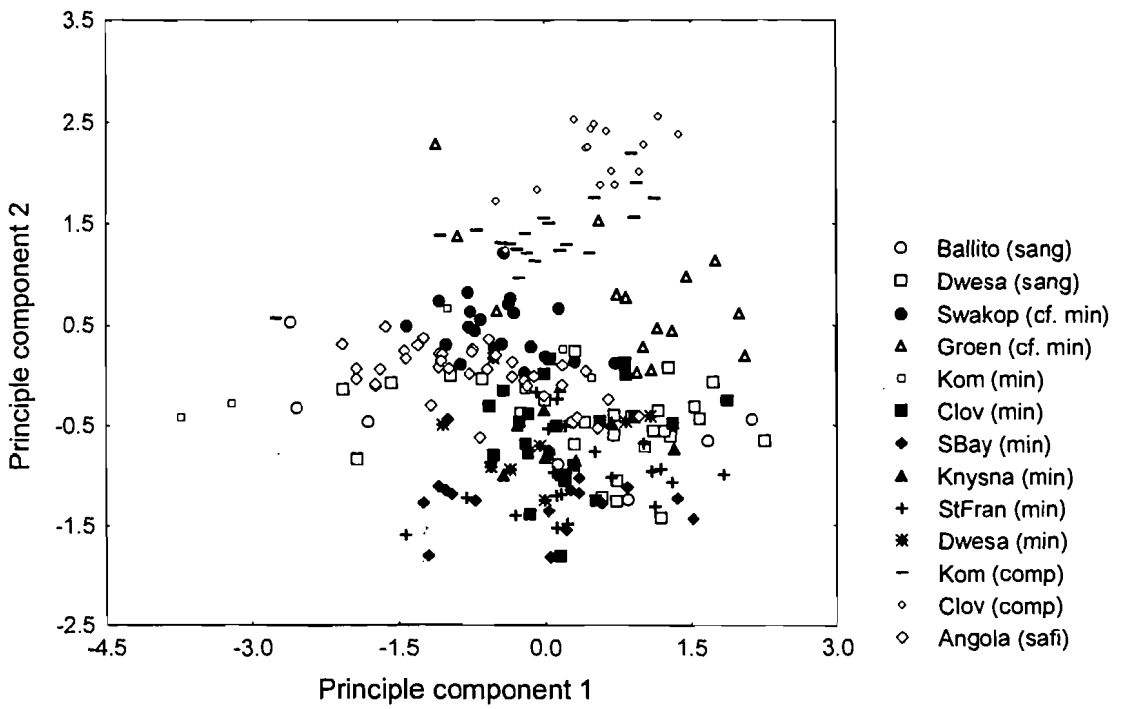


Figure 5.4 *Patella* cf. *miniata*, *P. miniata miniata*, *P. miniata sanguinans*, *P. compressa* and *P. safiana*. Scatterplot of the first two principle components based on the five shell morphometric characters. See Methods for site and species abbreviations.

sanguinans and *P. safiana* groupings, as indicated by the overlap of the convex hulls. However, *P. safiana* could be partly differentiated from *P. miniata miniata* and *P. miniata sanguinans* along the axis for the second canonical variable. Nevertheless, the *P. compressa* group clustered out completely from the other three groups. Most of the separation between the groups was along the axis of the first canonical variable, which accounted for about 74% of the total variance. The second canonical variable accounted for about 20% of the total variability, thus the first two canonical variables account for as much as 94% of the total variance. The standardised coefficients of the canonical variables indicated that separation along the first axis was largely due to shell width, whereas along the second axis, the separation was largely due to shell dry weight. To evaluate taxon differences, the proportion of correctly re-classified specimens in each taxa was calculated (Table 5.3). The classification function indicated that 81.82% of the individuals could be correctly identified. The *P. miniata miniata* and *P. compressa* groupings were correctly reassigned to their respective groupings with >90% accuracy, whereas *P. safiana* was correctly reassigned with 67% accuracy, and the *P. miniata sanguinans* group was correctly reassigned with <50% accuracy. Thus, it appears that a some differentiation is evident from the shell morphometrics, and that *P. compressa* in particular, being narrower than the other three species, clusters out from the other three groupings.

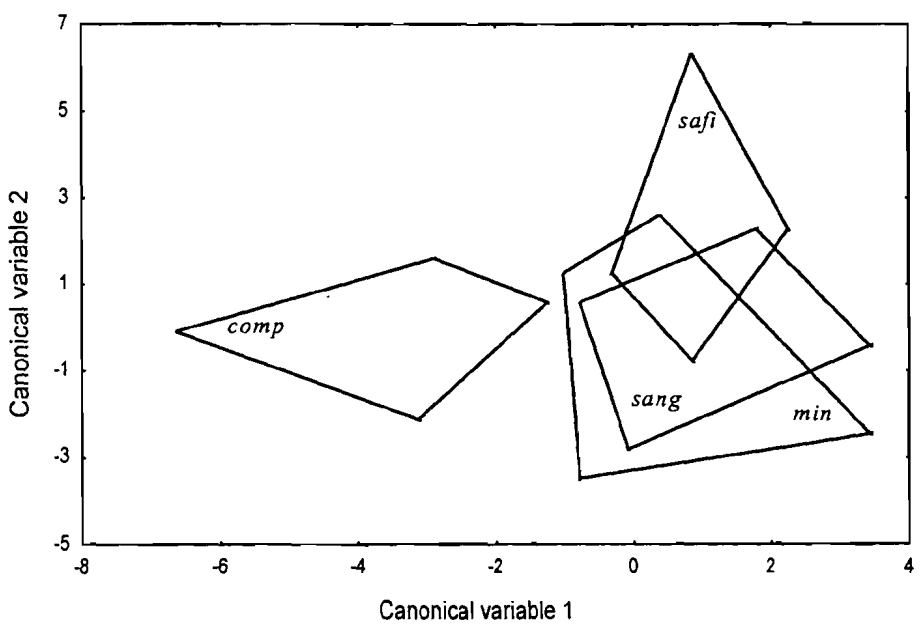


Figure 5.5 *Patella* cf. *miniata*, *P. miniata miniata*, *P. miniata sanguinans*, *P. compressa* and *P. safiana*. Plot of the first two canonical variables based on the five shell morphometric characters. See Methods for species abbreviations.

Table 5.3 Percent correct a posteriori classification to groups based on morphometric classification functions of shell variables

Group	No. of individuals				% Correctly classified
	A	B	C	D	
A *Patella miniata sanguinans*	17	18	0	1	47.22
B *Patella miniata miniata*	6	113	1	2	92.93
C *Patella compressa*	0	2	34	0	94.44
D *Patella safiana*	0	12	0	25	67.56

Electrophoretic analyses

The allele frequencies for the polymorphic loci and those showing fixed allele differences for all the populations of *Patella* cf. *miniata*, *P. miniata miniata*, *P. miniata sanguinans*, *P. compressa* and *P. safiana* are shown in Table 5.4. Seven of the loci were consistently monomorphic for all populations (ARK-2, SDH-1, HEX-1, ME-2, IDH-2, SOD-1, ALD-1), with SOD-1 and ALD-1 being fixed for different alleles in different taxa. The locus ALD-1 was fixed for allele ALD-1^B for *P. miniata sanguinans*, and for allele ALD-1^A in the other taxa. The locus SOD-1 was fixed for allele SOD-1^B for *P. compressa* and for allele SOD-1^A for the other taxa. Nine loci were polymorphic, with the number of alleles per locus ranging from two (ME-1, MDH-1, MDH-2) to four (GL-2, LGG-1, IDH-1), however, none of these loci were consistently polymorphic in every population. Four alleles coded for GL-2, but only one allele (GL-2^C) was shared by the majority of the populations: alleles GL-2^A and GL-2^B were unique to *P.* cf. *miniata*, *P. miniata miniata* and *P. compressa*; *P. miniata sanguinans* was fixed for allele GL-2^C, and *P. safiana* was uniquely fixed for allele GL-2^D. *Patella safiana* was also uniquely fixed for alleles LGG-1^D and IDH-1^D. GOT-1 showed differentiation between the populations in that *P. miniata sanguinans* and *P. compressa* had a predominance of allele GOT-1^B whereas *P.* cf. *miniata* and *P. miniata miniata* possessed a predominance of allele GOT-1^A. *Patella safiana* was once again uniquely fixed for a separate allele, allele GOT-1^C.

Table 5.4 *Patella* cf. *miniata* (cf. *min*), *P. miniata miniata* (*min*), *P. miniata sanguinans* (*sang*), *P. compressa* (comp), and *P. safiana* (*safi*). Distribution of allele frequencies at 11 loci in 13 populations. (N) = sample size

Locus	Swakopmund (cf. *min*)	Groen River (cf. *min*)	Kommetjie (*min*)	Clovelly (*min*)	Still Bay (*min*)	Knysna (*min*)	St Francis Bay (*min*)	Dwesa (*min*)	Dwesa (*sang*)	Ballito (*sang*)	Kommetjie (*comp*)	Clovelly (*comp*)	Angola (*safi*)
GPI-1													
(N)	20	16	12	25	19	12	20	8	28	9	20	20	25
A	0.300	0.781	0.667	0.600	0.789	0.542	0.525	0.938	0.839	0.833	0.925	1.00	0.060
B	0.700	0.219	0.333	0.400	0.211	0.458	0.475	0.063	0.161	0.467	-	-	0.940
C	-	-	-	-	-	-	-	-	-	-	0.075	-	-
PGM-1													
(N)	20	16	12	25	19	12	20	8	28	9	18	20	25
A	0.850	0.656	0.667	0.780	1.000	0.917	0.725	0.625	0.339	0.444	0.806	0.750	0.820
B	0.150	0.313	0.333	0.040	-	0.042	0.050	0.250	0.018	-	0.083	-	-
C	-	0.031	-	0.180	-	0.042	0.225	0.125	0.643	0.556	0.111	0.250	0.180
GL-2													
(N)	20	16	12	25	19	12	20	8	28	9	20	20	25
A	0.275	0.500	0.250	0.420	0.763	0.458	0.500	0.188	-	-	0.500	0.200	-
B	0.075	0.031	0.042	0.300	0.079	0.458	0.400	0.250	-	-	0.075	0.100	-
C	0.650	0.469	0.708	0.280	0.158	0.083	0.100	0.563	1.000	1.000	0.425	0.700	-
D	-	-	-	-	-	-	-	-	-	-	-	-	1.000
LGG-1													
(N)	20	16	12	25	19	12	20	8	28	9	20	20	25
A	1.000	1.000	1.000	1.000	1.000	1.000	0.875	1.000	0.179	0.833	0.950	0.925	-
B	-	-	-	-	-	-	0.100	-	0.821	0.167	0.025	0.050	-
C	-	-	-	-	-	-	0.025	-	--	-	0.025	0.025	-
D	-	-	-	-	-	-	-	-	--	-	-	-	1.000
ME-1													
(N)	20	16	12	25	19	12	20	8	28	9	20	20	25
A	0.950	0.875	0.625	0.780	0.526	0.750	0.500	1.000	1.000	1.000	0.550	0.500	1.000
B	0.050	0.125	0.375	0.220	0.474	0.250	0.500	-	-	-	0.450	0.500	-
GOT-1													
(N)	20	16	12	25	19	12	20	8	28	9	20	20	25
A	1.000	1.000	1.000	0.920	0.921	0.917	0.950	0.625	0.036	--	0.100	-	-
B	-	-	-	0.080	0.079	0.083	0.050	0.375	0.964	1.000	0.900	1.000	-
C	-	-	-	-	-	-	-	-	-	-	-	-	1.000

Table 5.4 (contd.)

IDH-1													
(N)	20	16	12	25	19	12	20	8	28	9	20	20	25
A	0.925	0.969	1.000	0.960	0.947	1.000	1.000	1.000	0.946	1.000	0.925	0.925	-
B	-	0.031	-	-	-	-	-	-	0.036	-	-	0.025	-
C	0.075	-	-	0.040	0.053	-	-	-	0.018	-	0.075	0.050	-
D	-	-	-	-	-	-	-	-	-	-	-	-	1.000
MDH-1													
(N)	20	16	12	25	19	12	20	8	28	9	20	20	25
A	0.900	0.969	0.958	0.980	0.947	1.000	1.000	0.938	0.929	0.722	1.000	0.900	1.000
B	0.100	0.031	0.042	0.020	0.053	-	-	0.063	0.071	0.278	-	0.100	-
MDH-2													
(N)	20	16	12	25	19	12	20	8	28	9	20	20	25
A	1.000	1.000	0.917	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-	-	0.083	-	-	-	-	-	-	-	-	-	-
ALD-1													
(N)	20	16	12	25	19	12	20	8	28	9	20	20	25
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	1.000	1.000	1.000
B	-	-	-	-	-	-	-	-	1.000	1.000	-	-	-
SOD-1													
(N)	20	16	12	25	19	12	20	8	28	9	20	20	25
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	1.000
B	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-

Tests for Hardy-Weinberg equilibrium (using Levene's (1949) correction for small sample size) indicated that of the 72 cases of polymorphism encountered at all loci in all populations, 25 (34.7%) exhibited significant deviations (χ^2, $p<0.05$). When values of genetic variation were averaged over species, *P. compressa* was the most polymorphic taxa for the loci studied (40.6% of loci studied were polymorphic), followed by *P.* cf. *miniata* 37.5%, *P. miniata miniata* 36.5%, *P. miniata sanguinans* 31.3%, with *P. safiana* showing the lowest value of 12.5%.

Genetic identity (*I*) and distance (*D*) values (Nei, 1978) were calculated for each pairwise population comparison (Table 5.5). The cluster analysis (Fig. 5.6) based on Nei's (1978) *I* revealed four distinctive groupings. The populations of *P.* cf. *miniata* and *P. miniata miniata* clustered together and had a mean intraspecific identity value of 0.979. The *P. compressa* populations yielded an intraspecific *I* value of 0.994, whereas the *P. miniata sanguinans* populations clustered together at a mean intraspecific *I* value of 0.970. *Patella safiana* clustered out by itself to form the fourth grouping. The *P. compressa* group clustered from the *P. miniata miniata* group at an *I* value = 0.851, and the *P. miniata sanguinans* group clustered out at an *I* value = 0.797. *Patella safiana* was the most differentiated and separated at an *I* value = 0.691.

Using the allele as the character in the phylogenetic analysis, 18 alleles were synapomorphic and were thus phylogenetically informative. Table 5.6 shows the distribution of these characters among the species. *Patella miniata miniata* and *P.* cf. *miniata* clustered together in Fig. 5.6 at a level indicating conspecificity, therefore *P.* cf. *miniata* is lumped under *P. miniata miniata* in the phylogenetic analysis. The analysis using the 'ie' command in the HENNIG86 programme yielded a single cladogram (Fig. 5.7a), with a CI of 1.00 and RI of 1.00. The characters 3-13, 15, 16 and 18 (Table 5.6) define *P. miniata miniata, P. miniata sanguinans* and *P. compressa* into a monophyletic group. It is evident that *P. compressa* and *P. miniata miniata* are more closely related to each other, than either of them is to *P. miniata sanguinans*. The close relationship between *P. compressa* and *P. miniata miniata* is supported by the characters 1, 2, 14 and 17 (Table 5.6). The analysis was also carried out using *P. granularis*, a more

Table 5.5 *Patella* cf. *miniata* (cf. *min*), *P. miniata miniata* (*min*), *P. miniata sanguinans* (*sang*), *P. compressa* (*comp*), and *P. safiana* (*safi*). Matrix of Nei's (1978) genetic identity (above diagonal) and genetic distance (below diagonal) averaged over 16 loci in 13 populations.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Swakopmund (cf. *min*)	*****	0.981	0.984	0.985	0.955	0.977	0.964	0.962	0.771	0.817	0.828	0.810	0.750
2. Groen River (cf. *min*)	0.019	*****	0.995	0.992	0.981	0.982	0.975	0.987	0.783	0.825	0.862	0.838	0.714
3. Kommetjie (*min*)	0.016	0.005	*****	0.987	0.974	0.976	0.976	0.987	0.783	0.825	0.862	0.838	0.714
4. Clovelly (*min*)	0.016	0.008	0.013	*****	0.987	1.000	0.995	0.980	0.781	0.824	0.866	0.844	0.738
5. Still Bay (*min*)	0.046	0.019	0.026	0.013	*****	0.987	0.987	0.956	0.743	0.789	0.876	0.844	0.704
6. Knysna (*min*)	0.023	0.018	0.025	0.000	0.013	*****	0.997	0.968	0.755	0.798	0.860	0.831	0.738
7. St Francis Bay (*min*)	0.036	0.026	0.025	0.006	0.013	0.003	*****	0.953	0.758	0.790	0.856	0.830	0.728
8. Dwesa (*min*)	0.038	0.013	0.022	0.020	0.045	0.032	0.048	*****	0.839	0.884	0.892	0.884	0.715
9. Dwesa (*sang*)	0.260	0.244	0.246	0.248	0.297	0.282	0.277	0.175	*****	0.970	0.766	0.788	0.626
10. Ballito (*sang*)	0.202	0.192	0.192	0.194	0.237	0.225	0.236	0.123	0.030	*****	0.809	0.831	0.631
11. Kommetjie (*comp*)	0.189	0.148	0.153	0.143	0.133	0.151	0.156	0.114	0.267	0.212	*****	0.994	0.629
12. Clovelly (*comp*)	0.210	0.177	0.168	0.170	0.169	0.185	0.186	0.123	0.238	0.185	0.006	*****	0.614
13. Angola (*safi*)	0.288	0.337	0.347	0.304	0.351	0.303	0.317	0.335	0.461	0.469	0.464	0.488	*****

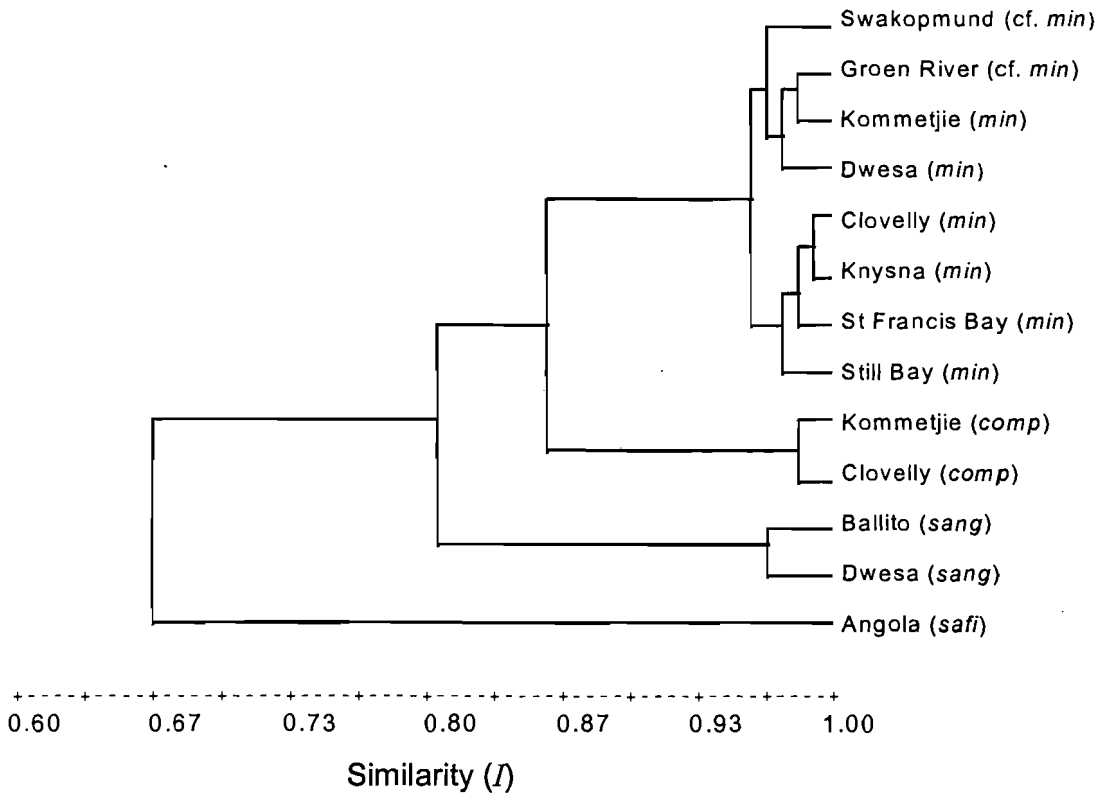


Figure 5.6 *Patella* cf. *miniata*, *P. miniata miniata*, *P. miniata sanguinans*, *P. compressa* and *P. safiana*. UPGMA dendrogram derived from Nei's (1978) genetic identity based on 16 loci. See Methods for species abbreviations.

distant relative to the species in question, as the outgroup, and an identical tree was obtained (CI = 1.00, RI = 1.00).

Using the locus as the character, only seven loci yielded phylogenetically informative characters. The data matrix of the allozyme character states are shown in Table 5.7. The analysis using the 'ie' command yielded a single cladogram (Fig. 5.7b), with a CI of 1.00 and RI of 1.00. The cladogram obtained in Fig. 5.7b is identical to that in Fig. 5.7a. Thus, the cladogram once again shows that *P. miniata miniata*, *P. miniata sanguinans* and *P. compressa* form a monophyletic group, supported by the synapomorphies in characters 2, 3, 5, and 7 (Table 5.7). The close relationship between *P. compressa* and *P. miniata miniata* are supported by the characters 1, 4 and 6 (Table 5.7). Once again, using *P. granularis* as the outgroup yielded identical results (CI = 1.00, RI = 1.00).

Thus, the electrophoretic and phylogenetic analyses clearly show that, although *P. miniata miniata*, *P. miniata sanguinans* and *P. compressa* are closely related, they differ genetically sufficiently to be recognised as separate species.

Morphological analyses

Shell and soft-part morphology: The shells of the *P. miniata miniata* grouping (i.e. *P.* cf. *miniata* and *P. miniata miniata*, Fig. 5.8a) showed a fair amount of individual intraspecific variation. The shell form was largely oval, reddish brown to pink in colour, and slightly narrowed anteriorly. The outer edge of the shell varied from being relatively smooth to having rather strong corrugations. The individuals from Groen River were collected from amongst the mussel beds and therefore had slightly higher shells in relation to the other populations. The underside coloration of the shell ranged from a pale blue-grey with the external pattern showing through very lightly as fine rays towards the margin for the *P.* cf. *miniata* populations, to a silvery pinkish white with the external pattern showing through strongly as fine pink rays for some of the *P. miniata miniata* populations. The colour of the foot was consistently grey underneath and purple on the side for all populations in this grouping. The head and cephalic tentacles were purple, with the mouth being a creamy-grey colour.

Patella miniata sanguinans showed little variation. Shells (Fig. 5.8b) were oval and slightly narrowed anteriorly. The external coloration was speckled brown with thick brown radiations from the apex. The underside coloration was silvery white with the brown external pattern showing strongly through as thick rays. The colour of the foot was a brownish-grey colour underneath and purple on the side. The head was purple, the cephalic tentacles a dark reddish purple, and the mouth creamy-grey.

The shells of *P. compressa* (Fig. 5.8c) were elongate-ovate, thin, tall and narrow with parallel sides. The sculpture of the shell consisted of numerous fine costae and the margin was very minutely crenulated. Externally the shell was a dull brownish to cream colour, and the underside a pearl grey colour. The colour of the foot was grey underneath and

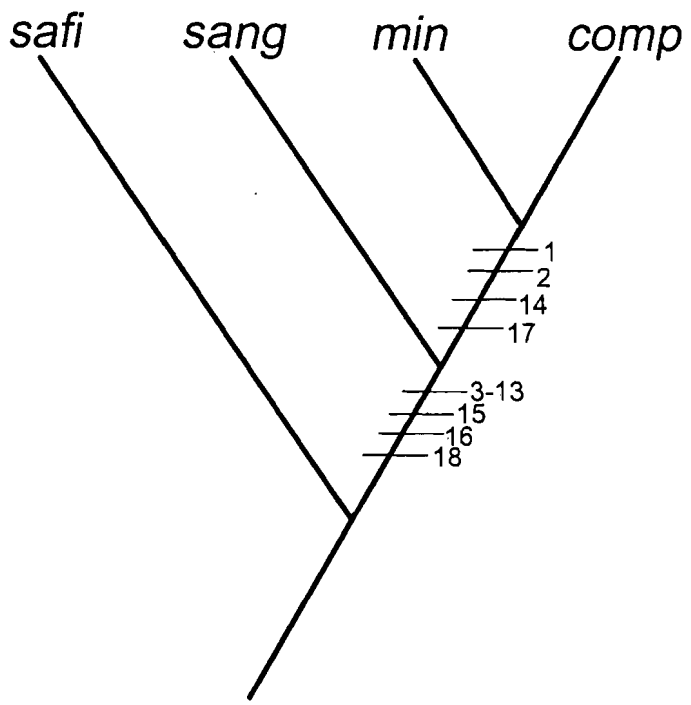
Table 5.6 Data matrix for using the allele as the character, showing the presence or absence of the characters for each species. All autapomorphs have been removed. See Methods for species abbreviations.

Locus/allele		character number	*min*	*sang*	*comp*	*safi*
GL-2	A	1	1	0	1	0
	B	2	1	0	1	0
	C	3	1	1	1	0
	D	4	0	0	0	1
GOT-1	A	5	1	1	1	0
	B	6	1	1	1	0
	C	7	0	0	0	1
IDH-1	A	8	1	1	1	0
	B	9	1	1	1	0
	C	10	1	1	1	0
	D	11	0	0	0	1
LGG-1	A	12	1	1	1	0
	B	13	1	1	1	0
	C	14	1	0	1	0
	D	15	0	0	0	1
MDH-1	B	16	1	1	1	0
ME-1	B	17	1	0	1	0
PGM-1	B	18	1	1	1	0

Table 5.7 Data matrix for using the locus as the character, with the states ordered using the *minimum turnover model* (Mickevich and Mitter, 1983), showing the character states for each species. See Methods for species abbreviations.

Locus	Character number	*min*	*sang*	*comp*	*safi*
GL-2	1	2	1	2	0
GOT-1	2	1	1	1	0
IDH-1	3	1	1	1	0
LGG-1	4	2	1	2	0
MDH-1	5	1	1	1	0
ME-1	6	1	0	1	0
PGM-1	7	1	1	1	0

a)



b)

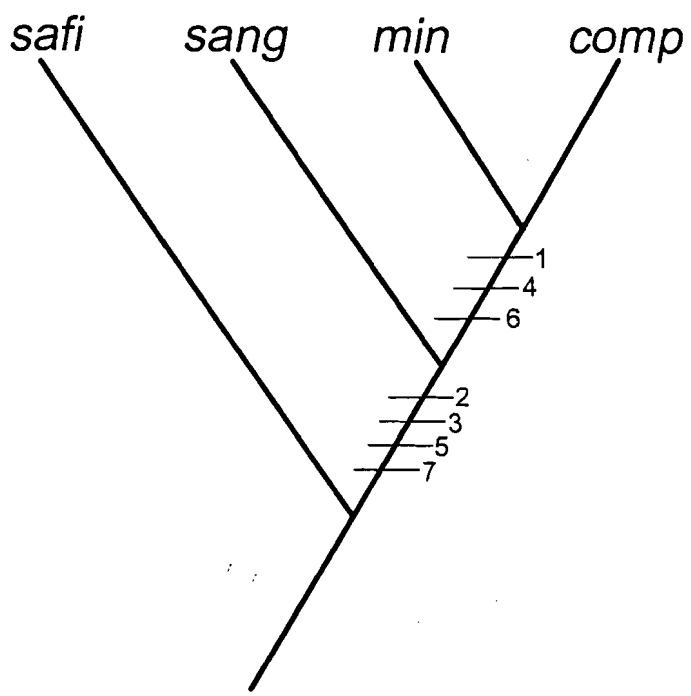


Figure 5.7 Consensus cladogram produced from the analysis illustrating the hypothesised relationships between *P. miniata miniata*, *P. miniata sanguinans* and *P. compressa* using (a) the allele as the character, and (b) the locus as the character. Bars indicate synapomorphies with the numbers referring to the character. See Methods for species abbreviations.

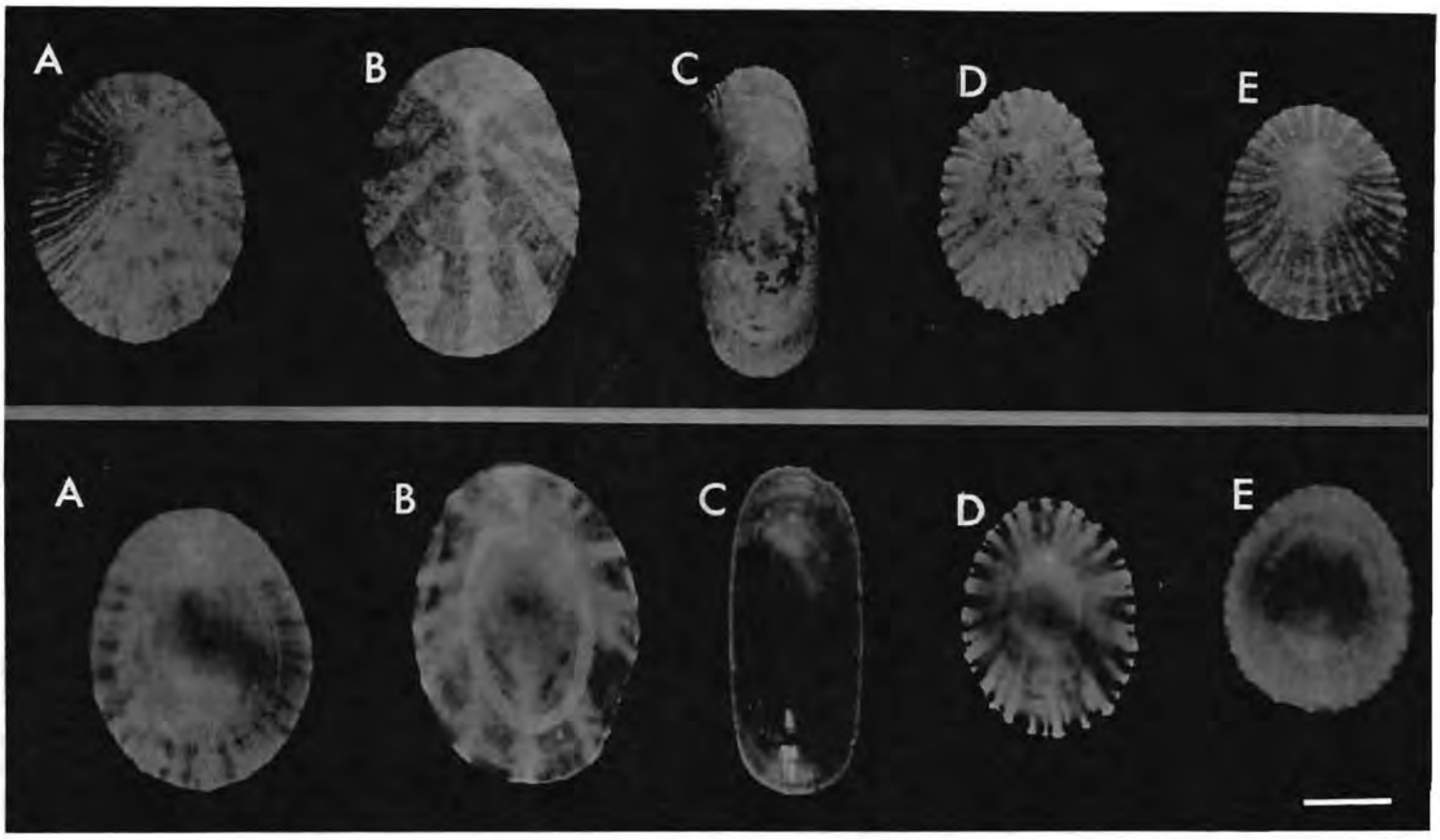


Figure 5.8 Dorsal and ventral views of the shells of (a) *P. miniata miniata*, (b) *P. miniata sanguinans*, (c) *P. compressa*, (d) *P. safiana*, and (e) the type specimen of *P. adansonii*. Scale bar: 17.5mm.

purple on the side for all populations in this grouping. The head and cephalic tentacles were purple, with the mouth being a grey colour.

Patella safiana shells (Fig. 5.8d) were oval and slightly narrowed anteriorly. The external coloration was whitish with conspicuous brown rays. The underside coloration was silvery grey with the dark brown to black external rays showing strongly through. The colour of the foot was brownish-grey underneath and purple grey on the side. The head and cephalic tentacles were purple, and the mouth a creamy-grey colour.

Radular morphology: Figure 5.9 shows representative samples of the radular structure in *P. miniata miniata*, *P. miniata sanguinans*, *P. compressa* and *P. safiana*. All four taxa had radula of similar structure described by the formula 3 + 1 + (2 + 1 + 2) + 1 + 3 (Powell, 1973). In all four taxa, the radulae had three pairs of unmineralised marginal teeth, three pairs of lateral teeth and a single, central rachidian tooth (Fig. 5.9). The lateral teeth were divided into a pair of large pluricuspid teeth and two pairs of smaller unicuspid teeth. The unicuspid teeth lie below each other, with the pluricuspid teeth lying below them, forming an inverted 'V' shape, as is characteristic of all the *Cymbula* clade (Koch, 1949). The pluricuspid tooth has four cusps (numbered from the outside to the centre of the radula), with cusps 2 and 3 being pointed and larger than the much reduced cusps 1 and 4. Despite the arrangement of the teeth being identical, *P. compressa* differed from the other taxa in that it had straight to concave cutting edges on the cusps whereas *P. miniata miniata* and *P. miniata sanguinans* had convex cutting edges. All three of these species had lateral cusps that rolled back on themselves, creating the appearance of the teeth having a hollow centre. This feature was absent in *P. safiana* and, indeed, appears in no other *Patella* species (G. Branch pers. comm.)

Gut looping: In all four taxa the X loop of the gut was coiled anticlockwise (see Ridgway, 1994). The Y loop was greater than 30% of the length of the X loop, and was therefore classified as being 'relatively long' in all four taxa. The mean and standard error of the ratios measured from the X and Z loops for each of the four taxa are shown in Fig. 5.10. The length of the Z loop relative to the visceral mass (Fig. 5.10a) shows that individuals of *P. miniata sanguinans* had relatively shorter Z loops than the other three, with

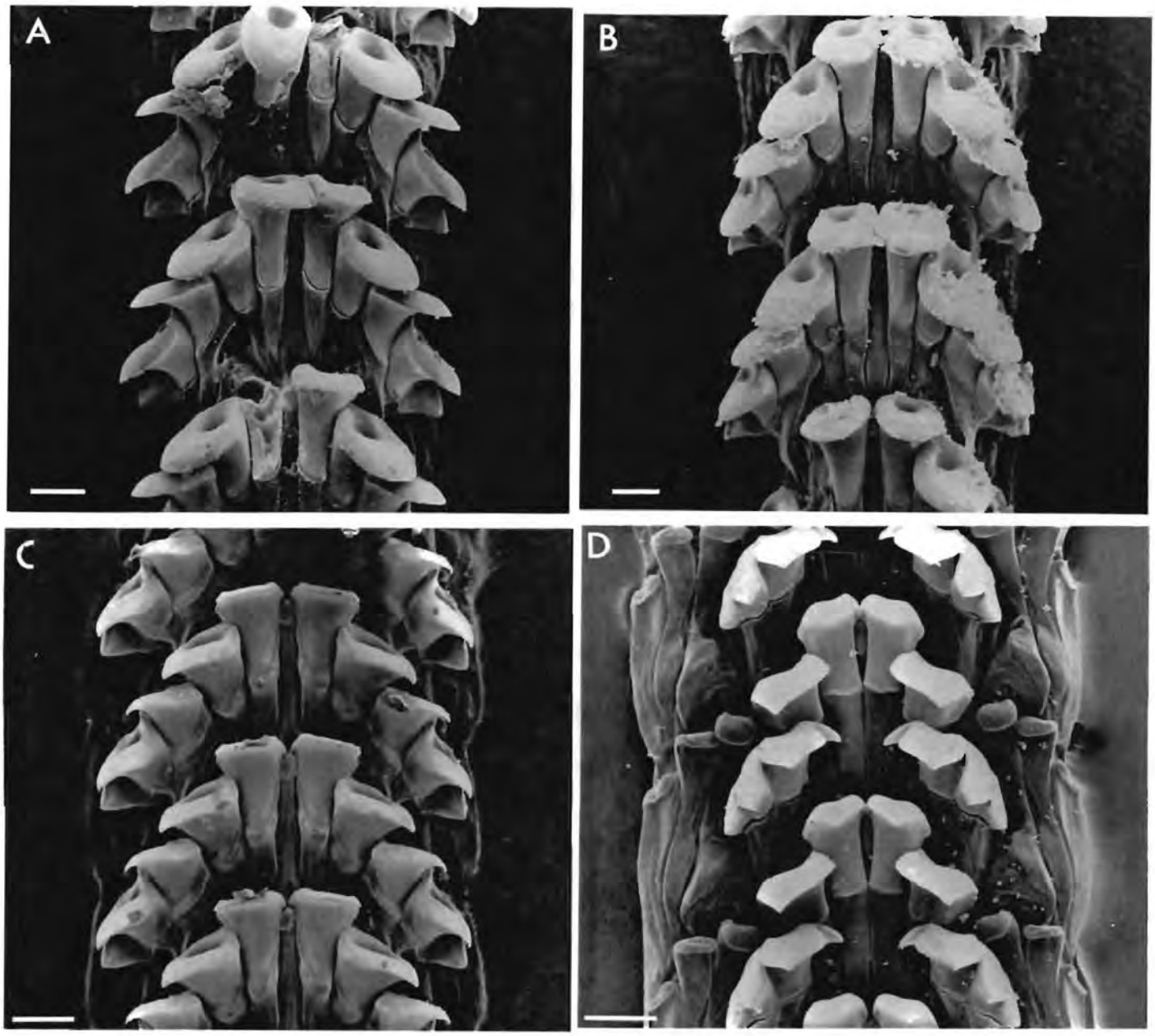


Figure 5.9 Scanning electron micrograph of the radular morphology of (a) *P. miniata miniata*, (b) *P. miniata sanguinans*, (c) *P. compressa*, and (d) *P. safiana*. Scale bar: 100μm.

P. compressa having the longest Z loops. *Patella miniata sanguinans* differed significantly (Kruskal-Wallis, $p<0.05$) from *P. miniata miniata* and *P. compressa*. *Patella safiana* also differed significantly from *P. compressa* (Kruskal-Wallis, $p<0.05$). The length of the X loop relative to the visceral mass (Fig. 5.10b) showed that *P. miniata miniata* has a longer X loop, differing significantly (Kruskal-Wallis, $p<0.05$) from *P. miniata sanguinans* and *P. compressa* for this character. The width relative to the length of the X loop (Fig. 5.10c) showed no significant differences (Kruskal-Wallis, $p>0.05$) between all four taxa.

Sperm microstructure: Sperm from all the taxa examined were of similar shape, all having nuclei which intrude into the subacrosomal space and possessing complex acrosomes, therefore being classified as Type III sperm (Fig. 5.11; Hodgson *et al.*, 1996). In addition according to Hodgson *et al.* (1996), *P. safiana* has sperm of a similar shape to the taxa examined. Table 5.8 shows the means and standard errors of the four measurements taken from the sperm. *Patella miniata miniata* had significantly longer sperm (total length of nucleus and acrosome), longer nuclei, as well as longer and narrower acrosomes (ANOVA $p<0.05$) than *P. miniata sanguinans* and *P. compressa*. In addition, the total sperm length and the length of the nucleus of *P. compressa* is significantly longer (ANOVA, $p<0.05$) than *P. miniata sanguinans*. Thus, the sperm microstructural dimensions provide useful information to distinguish between *P. miniata miniata*, *P. miniata sanguinans* and *P. compressa*.

Table 5.8 Sperm dimensions (mean ± SE) of *Patella miniata sanguinans*, *P. miniata miniata*, and *P. compressa*.

	Total length of nucleus and acrosome (μm)	Nucleus length (μm)	Acrosome length (μm)	Acrosome width (μm)
Patella miniata sanguinans	2.37±0.01	1.64±0.01	1.10±0.01	0.86±0.01
Patella miniata miniata	2.83±0.01	1.94±0.01	1.33±0.01	0.78±0.00
Patella compressa	2.43±0.01	1.70±0.02	1.11±0.01	0.84±0.01

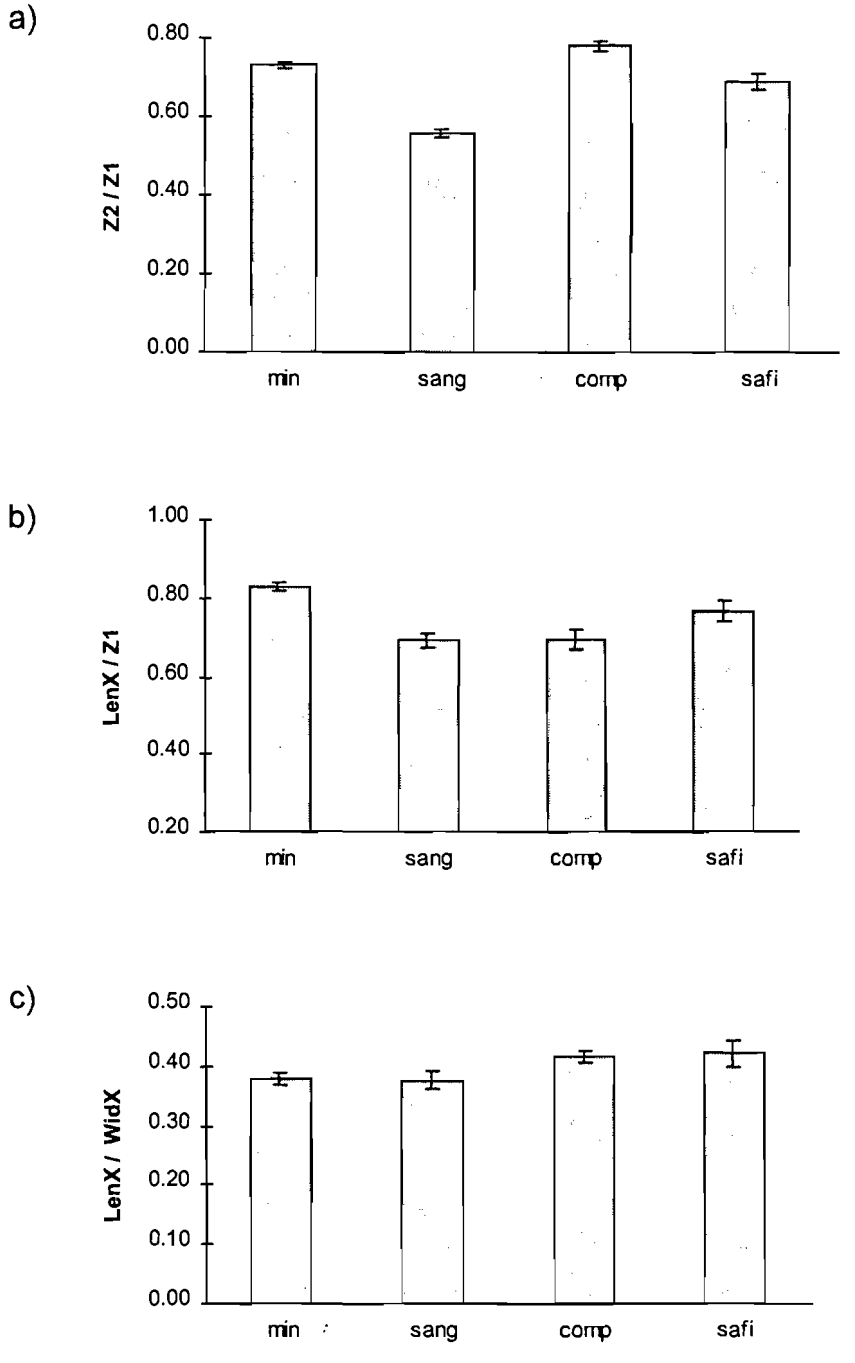


Figure 5.10 Gut loop measurements (mean ± SE) for *P. miniata miniata*, *P. miniata sanguinans*, *P. compressa* and *P. safiana*. See Figure 5.2 for interpretation of abbreviations.

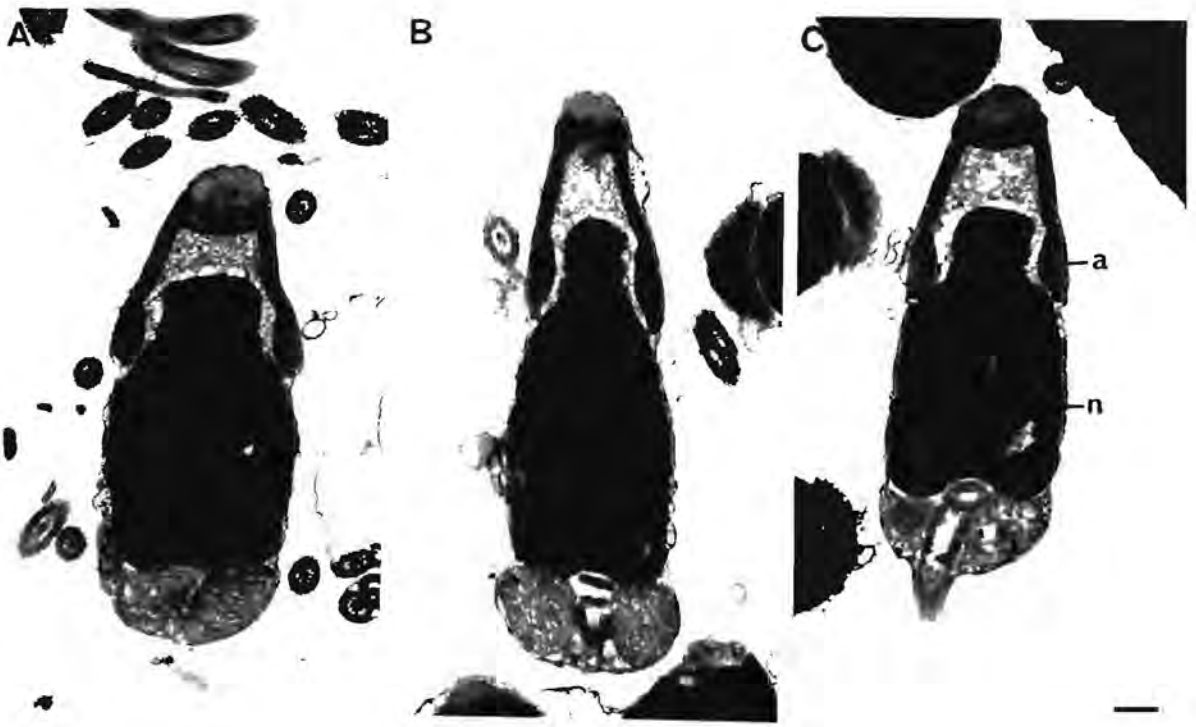


Figure 5.11 Mid-longitudinal TEM sections through the spermatozoa of (a) *P. miniata sanguinans*, (b) *P. miniata miniata*, and (c) *P. compressa*. a, acrosome; n, nucleus. Scale bar: 200nm.

DISCUSSION

Patella miniata sanguinans shows sufficient differences from *P. miniata miniata* to warrant full specific status. Its populations cluster out from those of *P. miniata miniata* at a genetic identity value of 0.797, a value typical of congeneric species. However, of greater significance is the presence of a fixed allele difference at ALD-1 between the two taxa. *Patella miniata sanguinans* and *P. miniata miniata* were found sympatrically at Dwesa, and the presence of this fixed allele difference, and the fact that no intermediates were found between the two, demonstrates a lack of gene flow and therefore indicates that they are reproductively isolated.

In addition, the morphological data largely mirror the genetic differences between the two. The external coloration of the shells of *P. miniata sanguinans* is speckled brown with thick brown radiations originating from the apex, whereas the shells of *P. miniata miniata* are reddish brown to pink in colour, with radiations that are fine or even absent. The underside of the foot coloration also shows differences in that *P. miniata sanguinans* has a browny-grey colour whereas that of *P. miniata miniata* is a purple grey. Further differences were evident in the gut-loop coiling, in that *P. miniata sanguinans* had significantly shorter X and Z loops than *P. miniata miniata*. Finally, sperm microstructural differences were detected between the two species, with *P. miniata miniata* having significantly longer and narrower sperm than *P. miniata sanguinans*.

A further interesting result was observed in the behaviour of *P. miniata sanguinans*, in that at both Dwesa and Ballito, individuals were 'gardening' algal resources. 'Gardening' can be defined as the 'modification of plant assemblages, caused by the activities of an individual grazer within a fixed site, which selectively enhances particular plant species and increases the food value of the plants for the grazer' (Branch *et al.*, 1992). The 'gardens' vary in size depending on the size of the limpet, but the mean areas for the 'gardens' were 994.5 cm^2 (±139.3) and 462.9 cm^2 (±112.8) for Dwesa and Ballito respectively. The algae associated with the 'gardens' were *Peyssonnelia atroppurpurea* at Dwesa, and *Ralfsia verrucosa* at Ballito. Thus, 'gardens', although depressing algal species richness, increase local productivity relative to adjacent areas because continual

grazing maintains the algal species in an early, rapid phase of growth. No suggestion of 'gardening' was observed in *P. miniata miniata*. Therefore, in this respect *P. miniata sanguinans* and *P. miniata miniata* differ in behaviour. However, no data are available on whether or not the individuals of *P. miniata miniata* are 'gardening' algal resources at Dwesa (where the two species are sympatric), so that it is difficult to conclude whether this is a fixed difference, or merely a response of the *P. miniata sanguinans* individuals to the lower productivity on the shores of the east coast.

The suggestions of Pilsbry (1891) and S. Ridgway *et al.* (in press) that *P. miniata* and *P. compressa* may be ecomorphs of the same species is not supported by my data. In both the PCA and the discriminant functions analysis, the populations of *P. compressa* cluster out from not only the *P. miniata miniata* populations, but also from the populations of *P. miniata sanguinans* and *P. safiana*. The *P. compressa* shells are easily distinguishable in that, due to the habitat of these limpets on the stipes of kelp (*Ecklonia maxima*), they have a totally different shape to the shells of *P. miniata miniata*. The shells of *P. compressa* are elongate, thin, tall and narrow with parallel sides, whereas those of *P. miniata miniata* are ovate. The radula of *P. compressa*, although having the same formula as *P. miniata miniata*, differs in the shape of the cusps, in that those of *P. compressa* have straight to concave cutting edges, whereas those of *P. miniata miniata* have convex cutting edges. The length of the X loop of *P. compressa* is also significantly shorter than that of *P. miniata miniata*, and the sperm of *P. compressa* is significantly shorter and broader than that of *P. miniata miniata*.

Furthermore, the populations of *P. compressa* cluster out from the *P. miniata miniata* populations at a genetic identity of 0.851. A fixed allele difference between *P. compressa* and *P. miniata miniata* was evident at SOD-1 for sympatric populations of these two species at Kommetjie and Clovelly, and no intermediates were detected between the two species at these sites. The presence of the fixed allele differences between them at these sites demonstrates a lack of gene flow, and thus, reproductive isolation between the two.

The genetic identities obtained in this study were similar to those obtained in other studies comparing different species. Thorpe (1982) proposed that the critical level for genetic

identity values distinguishing species and genera is about 0.35, with values between congeneric species falling between 0.35 and 0.85. Although Thorpe's proposed separation of species has been widely used and accepted in taxonomic studies, it is only a guide and should therefore be used with caution. Similar studies have calculated genetic identities of 0.64 between *P. caerulea* and *P. depressa* (Côrte-Real *et al.*, 1996a), and 0.63 between *P. aspera* and *P. ferruginea* (Cretella *et al.*, 1994). Other studies show genetic identity values of 0.58 (Côrte-Real *et al.*, 1996a) and 0.53 (Chapter 2) between species of the genus *Patella*. Côrte-Real *et al.* (1996b) recorded a genetic identity value of 0.86 between *P. rustica* and *P. piperata*, effectively recognising the two as separate species. Thus, if these published values and Thorpe's (1982) scale is taken into consideration, then *P. miniata sanguinans* and *P. compressa* should be recognised as valid congeneric species.

The populations from Swakopmund and Groen River in this study were initially identified as *P. adansonii* (following Ridgway *et al.*, in press), however, I was not certain whether these individuals were specimens of *P. miniata miniata* or specimens of *P. adansonii* (originally described from West Africa), and hence, they were called *P.* cf. *miniata* for the purposes of this study. However, in the electrophoretic analyses, the *P.* cf. *miniata* and *P. miniata miniata* populations cluster out in a distinctive group with a mean genetic identity of 0.979. This value is typical of conspecific populations (Thorpe, 1982; Côrte-Real *et al.*, 1996a; Chapter 2) and it was therefore concluded that the individuals of *P.* cf. *miniata* were in fact *P. miniata miniata*. Furthermore, the sperm of the populations of *P.* cf. *miniata* did not differ significantly from the *P. miniata miniata* populations. In addition, after examination of the original description (Dunker, 1853) and the type material of *P. adansonii* (Museum für Naturkunde der Humboldt-Universität zu Berlin 102150, 102151), I could not find any major differences between the shells of the individuals of *P.* cf. *miniata* from Swakopmund and Groen River and the type of *P. adansonii* (stated to have come from Angola). Thus, based on the genetic data in this study, and the fact that I cannot find any definitive morphological characteristics that distinguish the type of *P. adansonii* from *P. miniata miniata*, I propose that *P. adansonii* be synonomised with *P. miniata*.

Both of the cladograms produced from the allozyme data are identical (Fig. 5.7). Since the same data set was employed for the two methods used, it appears that the choice of coding was not too critical for this analysis. Amongst recent studies of the phylogeny of molluscs, Hoeh (1990) and Emberton (1995) use the allele as the character, Emberton (1994) and Bandoni, Mulvey and Loker (1995) use the locus as the character, whereas Dempster (1995) employed both the allele and the locus as characters. Dempster (1995) obtained cladograms that were consistent with each other, differing only in the degree of resolution. The results of the cladograms (Fig. 5.7) show that *P. miniata miniata*, *P. miniata sanguinans*, and *P. compressa* form a closely related monophyletic group. More interestingly, however, is that the results show that *P. miniata miniata* is more closely related to *P. compressa* then it is to *P. miniata sanguinans*. This therefore provides strong additional evidence that *P. miniata sanguinans* should be recognised as a full species rather than merely as a subspecies of *P. miniata*.

Thus, based on the evidence from this study, it can be concluded that three species are involved in the '*Patella miniata*' species complex in southern Africa. Suggestions that *P. miniata* and *P. compressa* may in fact be ecomorphs of the same species are not well founded, and both *P. miniata* and *P. compressa* remain as full species. Furthermore, *P. miniata sanguinans* is sufficiently different to warrant full specific status, and I therefore raise it from subspecific status to the full specific status, *P. sanguinans*. Thus, the '*Patella miniata*' species complex in southern Africa is a closely related monophyletic group of three species with *P. miniata* and *P. compressa* being more closely related to each other than either one is to *P. sanguinans*. Finally, following Ridgway *et al.* (in press), I recognise all three as members of the *Cymbula* clade and advocate that they be allocated to the genus *Cymbula* as distinct from the more narrowly defined genus *Patella*.

Chapter 6

Synthesis

Although the southern African representatives of the genus *Patella* are considered to be fairly well characterised, there were some biologically and taxonomically interesting questions that remained unanswered when I began this study, and it was upon these that I have concentrated in this thesis (Chapter 1).

Firstly, *Patella granularis* was considered to be the most widely distributed of all the southern African limpet species. However, it was shown that the east-coast individuals, which were originally identified as *P. granularis*, were sufficiently different genetically to warrant the recognition of two separate species. In particular, the genetic analysis revealed fixed-allele differences that persisted even in sympatry, therefore providing conclusive evidence that the two entities are reproductively isolated and constitute valid separate species. Furthermore, the genetic differences were mirrored by other morphological characters, and it was therefore concluded that the east-coast individuals previously identified as *P. granularis*, were not in fact this species, but rather represented a cryptic and closely related sibling species (Chapter 2). This species was recognised to be *P. natalensis*, originally described by Krauss (1848). In Chapter 3 a re-description is provided, together with a discussion of how it differs from *P. granularis* and *P. miliaris*.

Secondly, the population genetics of *P. barbara* were examined in Chapter 4 to assess whether morphological differences and the behavioural differences reported by Branch *et al.* (1992) were mirrored by genetic and/or morphological differences. In this process, an unusual variety of *P. barbara* was recorded on the tests of the ascidian *Pyura stolonifera* at Clovelly. This *Pyura*-dwelling form was much reduced and has taller shells compared to the 'normal' rock-dwelling form, therefore demonstrating the phenotypic morphological plasticity of the shells. Branch *et al.* (1992) have previously reported that in some populations, *P. barbara* undertakes 'gardening' by modifying the nature of the algal communities around individual limpets, increasing their value as a source of food in the process. They also hypothesised that the need for 'gardening' will be greatest in areas where productivity is low. I failed to detect any 'gardening' by *P. barbara* on the west

coast, where productivity is high, but recorded it at Arniston where productivity is lower, and found 'gardens' to be the largest and best-developed at Dwesa, which experiences the lowest productivity of all the sites examined. However, 'gardens' were absent at three other sites with moderately low productivity on the south coast. This therefore casts doubt on the idea that the phenomenon of 'gardening' is necessarily important in nutrient-poor waters.

However, possibly the most important result from this part of the study was the fact that there is very little genetic population differentiation in *P. barbara* along the coast of South Africa. This is particularly interesting because the genetic homogeneity of the populations indicates widespread gene flow, as would be expected from a broadcast spawner such as *P. barbara*. Marine gastropods exhibit different modes of larval development which potentially influence the extent of gene flow between populations (Grant and Utter, 1988). Electrophoretic studies have shown that species with long-lived planktonic larvae with considerable opportunity for passive dispersal by ocean currents have a high potential for gene flow that leads to genetic homogeneity (Grant and da Silva-Tatley, 1997). Thus, marine gastropods with pelagic larvae show genetic homogeneity over large portions of their ranges. The results from my study provide good examples for this model because all the species under question are broadcast spawners with a free-swimming larval stage. Chapter 2 shows that the populations of *P. granularis* are also genetically homogenous from Swakopmund in Namibia to Coffee Bay in the Transkei, a distance of over 2000 km. The populations of *P. barbara* in chapter 3 are largely genetically homogenous over a distance of some 1600 km. Chapter 4 shows that the populations of *P. miniata*, extending from Swakopmund in Namibia to Dwesa in the Transkei (approximately 2000 km), also show very little differentiation. Thus, the results obtained from the populations of *P. granularis*, *P. barbara* and *P. miniata* provide information on gene flow in southern African patellid limpets, in that the genetic homogeneity indicates widespread gene flow along the coast of southern Africa.

The final section of the thesis (Chapter 5) grapples with the taxonomic relationships between members of the '*P. miniata*' complex. This showed that the west-coast variety (*P.* cf. *miniata*) is genetically inseparable from *P. miniata* and synonymous with it, despite

differences in shell colour. In the process, the type specimen of *P. adansonii* was examined and found to fall within the range of variation of *P.* cf. *minata*, so that it was also sunk into *P. miniata*.

The suggestions of Pilsbry (1891) and Ridgway *et al.* (in press) that *P. compressa* and *P. miniata* may prove to be ecomorphs of the same species were not supported by my study. It was shown that there were sufficient differences pertaining to the genetics, shell dimensions, radular cusp shape and sperm microstructure of the two species to warrant full specific status for both. Thus, it was concluded that both *P. compressa* and *P. miniata* represent full species.

Prior to this study, *P. sanguinans* was considered by Kilburn and Rippey (1982) and Branch *et al.* (1994) to be a subspecies of *P. miniata*, and it was therefore called *P. miniata sanguinans*. Other authors (Koch, 1949; Christiaens, 1973; Powell, 1973) have gone further and simply synonomised it under *P. miniata*. However, this study conclusively showed that *P. miniata sanguinans* is in fact a valid species, and it was therefore raised to the full specific status, *P. sanguinans*, as described by Reeve (1854). The justification for this was once again based on the integration of genetic and morphological differences between *P. miniata miniata* and *P. miniata sanguinans*. Furthermore, the phylogenetic analysis of *P. miniata miniata*, *P. miniata sanguinans* and *P. compressa* supported the elevation of all three species to full specific status. The cladistic phylogeny showed that *P. miniata* and *P. compressa* are more closely related to each other than either of them is to *P. sanguinans*.

The development of molecular techniques in the past twenty to thirty years, has provided increased taxonomic clarity in a wide variety of molluscan taxa. Protein gel electrophoresis has proved to be a rapid and reliable method of assessment, which allows for the specific identification of separate gene pools, and is of considerable value in distinguishing species. This study has highlighted the strength and application of this technique, not only for systematic questions, but also for studies of population genetics. Furthermore, due to the great morphological plasticity of patellid limpets, full confidence cannot be placed on taxonomy that is based on the traditional morphological characters

alone. Thus, the use of both genetic and morphological characters in this study highlights the importance of integrating both molecular and traditional morphological methodologies in systematic studies.

In conclusion, this thesis has not only revealed patterns of gene flow and defined species boundaries within the genus *Patella* in southern Africa, but it has resolved some of the long-standing taxonomic disputes that have been associated with the South African representatives of this genus. Thus, taking the literature and the results of this study into consideration, twenty species can now be considered to be representative of the family Patellidae in South Africa. Although my thesis was not concerned with higher-level taxonomy and I used the generic name '*Patella*' to encompass all the species, it does seem that this group is too widely defined to reflect the clades evident within it (Ridgway *et al.*, in press). Following the four clades of Ridgway *et al.* (in press) (three of which occur in southern Africa), the southern African species can now be listed as:

The *Cymbula* clade:

- *Cymbula compressa* (Linnaeus, 1758)
- *C. granatina* (Linnaeus, 1758)
- *C. miniata* (Born, 1778)
- *C. oculus* (Born, 1778)
- *C. sanguinans* (Reeve, 1854)

The *Scutellastra* clade:

- *Scutellastra aphanes* (Robson, 1986)
- *S. argenvillei* (Krauss, 1848)
- *S. barbara* (Linnaeus, 1758)
- *S. cochlear* (Born, 1778)
- *S. flexuosa* (Quoy & Gaimard, 1834)
- *S. granularis* (Linnaeus, 1758)
- *S. longicosta* (Lamarck, 1819)

- *S. obtecta* (Krauss, 1848)
- *S. pica* (Reeve, 1854)
- *S. natalensis* (Krauss, 1848)
- *S. tabularis* (Krauss, 1848)

The *Helcion* clade:

- *Helcion concolor* (Krauss, 1848)
- *H. dunkeri* Krauss, 1848
- *H. pectunculus* Gmelin, 1791
- *H. pruinosus* (Krauss, 1848)

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