TAXONOMY, MORPHOLOGY AND POPULATION GENETICS OF MEDITERRANEAN *PATELLA*

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by

Calum Nobles B.Sc. Hons

(University of Liverpool)

Port Erin Marine Laboratory,
Department of Environmental and Evolutionary Biology,
School of Life Sciences,
University of Liverpool,
Port Erin,
Isle of Man,
British Isles.

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No information is missing

If you walk up and down a pebbly beach, you will notice that the pebbles are not arranged at random. The smaller pebbles typically tend to be found in segregated zones running along the beach, the larger ones in different zones ....A tribe living near the shore might wonder at this evidence of sorting .... and develop a myth to account for it

Richard Dawkins, The Blind Watchmaker, 1986

To My Torch

and

My Family

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Abstract

TAXONOMY, MORPHOLOGY AND POPULATION GENETICS OF MEDITERRANEAN *PATELLA*

Calum John Nobles

This work considers aspects of the taxonomy, population genetics and morphological variation of the genus *Patella* in the Mediterranean, concentrating principally on the Mediterranean endemic *P. caerulea*. Horizontal starch gel electrophoresis was used to determine the biochemical genetic composition of individuals. Qualitative information was recorded on shell form, a number of soft body characters and radula morphology. Multivariate statistical techniques were used to analyse morphometric variation based on seven shell measurements and radula length.

Infralittoral and mesolittoral groups of *P.caerulea* were compared where the species was found bimodally distributed. The two groups appeared to constitute a single genetic population , yet were morphologically distinct with an assignment rate in excess of 85% from a linear discriminant function. Infralittoral individuals tend to have a pentagonal shell outline (the widest point of the shell being towards the posterior and narrow across the shell apex), and a shorter radula, a shell with a more depressed shell and a greater curvature to the basal plane than do mesolittoral individuals. The implications of this stark contrast between genetic and morphometric results are discussed, with reference to the life history and behaviour of *P.caerulea*.

In samples of *P.caerulea* from throughout the Mediterranean all samples were viewed as constituting a single species. The genetic variation between limpets throughout the Mediterranean seems to be clinal, although there does appear to be some hydrographical barriers to gene flow, notably between Corsica and Northern Italy and between the eastern and western basins. It is probable that intermittent genetic exchange prevents divergence of the limpets from the two basins. The heterozygote deficiencies found in the samples suggest that there are complex interactions in the larval distribution and settlement of this species that affect the genetic make up of populations. The species shows high levels of intra-specific morphological variation, the differences in morphology are probably not dictated by geographic location but rather by habitat differences between the sample sites.

An investigation into the differences between *P.caerulea* and the morphologically similar Atlantic patellid *P.depressa* revealed them to be genetically distinct, with a number of diagnostic loci. The multivariate analysis gives a high level of discrimination between the two species, the problems of relying on such analysis are discussed in light of the environmental influences on shell shape. Possible reasons for the geographic distributions of the two species are given based on the inferred levels of gene flow between regions, it is suggested that current patterns limit the distribution of both species.

The intra- and inter specific morphological variation of the six species of *Patella* found in the Mediterranean is examined. Multivariate techniques allow the species to be distinguished on the basis of shell morphology with correct assignment levels of up to 100% from a linear discriminant function. The possible use of a linear discriminant function to identify individuals from subsequent samples is discussed. The differences in soft body characters, radula morphology and shell form that allow field identification of the six species are given. Field notes on the rare Mediterranean endemic *P.ferruginea*, and aspects of the ecology of this species that are in need of investigation are outline.

1- General Introduction

Limpets of the genus *Patella* play an important role in the ecology of the littoral zones they inhabit (Branch 1981; Branch 1984). Limpet grazing has a clear controlling effect on algal vegetation on exposed and moderately exposed shores in the North-east Atlantic (Hawkins 1981a, 1981b; Hawkins and Hartnoll 1983; Hawkins *et al.* 1992). The gardening patellids of South Africa have a marked effect upon their environment (Branch and Branch 1981; Branch 1984; Branch *et al.* 1992). Patellid limpets clearly play a very important role in determining the community structure on the shores they are found. The patellids in the Mediterranean appear to play an equally important role as in the Atlantic. The limited tidal range may serve, however, to concentrate their effect (Della Santina and Chelazzi 1991; Della Santina *et al.* 1992; Benedetti-Cecchi and Cinelli, *in press.*).

The abundance of the genus on the shore has naturally led to their exploitation by man as a food source. It is clear from archaeological evidence that patellids were collected from very early times (Cunliffe & Hawkins, 1988) and until recently they were eaten in Britain. Throughout the Mediterranean they are heavily exploited by the local population (Safiel *et al. in press*; C. Nobles *pers. obs.*). In the Azores, the genus supported an important fishery until the early 1980's when the stocks crashed (Martins *et al.* 1987).

Despite the importance of the genus, the taxonomy has been confused (Fischer-Piette 1935, 1948; Evans 1953; Christiaens 1973; Powell 1973). The conclusions drawn by the three most recent review works on patellid taxonomy give very conflicting pictures (Christiaens 1973; Powell 1973; Nordsieck 1982). There is clearly still uncertainty over the taxonomic status of some species of *Patella*. Additionally, little is known of the relatedness of geographically separated con-specifics (Côrte-Real 1992). Côrte-Real (1992) provides a good review of taxonomic works, and tends to agree with the taxonomic conclusions of Christiaens (1973).

This introduction will briefly outline the history of patellid taxonomy and the species origin models proposed for *Patella* in the Mediterranean and North-east Atlantic. The species of *Patella* found in the Mediterranean will be discussed. Also the techniques used in the investigation of taxonomy and population structure in *Patella* will be reviewed. An overview

of the geological history, geographic and vertical zonation of the Mediterranean will be given. Penultimately, more detailed species descriptions will be given for each of the Mediterranean species, with information on taxonomic synonyms; shell, radula and soft body morphology and geographic distributions. Finally after this review, the general aims of the thesis and specific aims of each chapter will be outlined.

1.0 - Limpet taxonomy

Much of the early work on taxonomy was based on the morphology of a limited number of specimens (e.g. Fischer-Piette 1935, see Christiaens 1973 for a review of such work). Patellids show extremely high intra-specific variation in both shell and soft body characters even between individuals in the same locale (Sella and Bacci 1971; Sella 1976; Côrte-Real 1992). This led to a profusion of species and sub-species being described by various early taxonomists (Gmelin 1791; Lamarck 1801; Lamarck 1819; Forbes 1853; Jeffreys 1865; Fischer 1880; Pilsbry 1891, 1894; Dautzenberg 1906, 1925).

Subsequently there was a tendency to regard the members of the genus as a single or small number of species with many varieties. Work by Jeffreys (1865) in England described *elevata*, *picta*, *intermedia*, *depressa* and *caerulea* as varieties of *P. vulgata*. Similarly, work in the Basque region of Spain (Fischer 1880) recognises only *P. vulgata* with five varieties: *athletica*, *lusitanica*, *caerulea*, *tarentina* and *scutellaris*.

Pilsbry's classification (Pilsbry 1891) divided the genus into two species: one from the Atlantic - *P. vulgata* (with varieties *elevata, picta, intermedia*, and *depressa*), the other from the Mediterranean - *P. caerulea* (with varieties *fragilis, intermedia, adspersa* and *subplana*). Further he suggested that *P. vulgata* and *P. caerulea* represented two extremes of a morphological cline and that a boundary between them is somewhat artificial. They could, on this basis, be regarded as one highly variable species. A more comprehensive study by Dautzenberg between 1887 and 1913 recognised four main species; *P. vulgata, P. intermedia, P. depressa* and *P. caerulea*. It should be noted that at this time no genetic mechanism for evolution was known and this is reflected by the ideas of 'varieties' of species.

An examination of patellid phylogeny by Fischer-Piette (1935, 1938, 1939, 1948, 1959) suggested that *P. vulgata* and *P. aspera* arose from a basic *depressa-caerulea* stock.

Evans (1953) suggested that Fischer-Piette's work was flawed due to the use of small sample sizes. He proposed a root stock of *P. vulgata* from which the other species arose. Another proposed origin was that of two stocks one comprising *P. vulgata-intermedia-caerulea-lugubris-candei* and the other comprising *P. aspera* alone (Christiaens 1973).

These "origin models" relied on the apparent existence of transitional forms. Later work however, showed that these so called transitional forms or hybrids were not genetically identifiable as such (Hatch 1977; Gaffney 1980). As there was no genetic difference between *P. vulgata* and transitional forms, the basis of the old theories on species origin were undermined.

Dodd (1957) successfully reared *Patella* hybrids to the metamorphosis stage. However as no *Patella* larvae have been successfully reared to settlement stage (Bowman 1981), there is no evidence that viable adult hybrids can be produced and there is no demonstration that viable adult hybrids occur in the 'wild' (Hatch 1977; Gaffney 1980).

These genetic studies demonstrate that traditional specific assignment, on the basis of shell morphology alone, is of fairly limited value in the light of the high levels of morphological variation occurring within a species. There are sufficient species-specific soft body characteristics to distinguish between the three British species. This however is considerably more difficult for Mediterranean and Macaronesian species due to their overlap in coloration and form.

1.1 - Limpets of the Mediterranean

There is confusion as to the number and names of patellid species present in the Mediterranean. Grandfils and Vega (1982) record the presence of *P. vulgata* along the coast of Malaga (Spain). Christiaens (1983) regards this as a misidentification resulting from work by Piani (1980) who includes *P. vulgata* as a Mediterranean species but omits *P. depressa*. It is Christiaens' contention that this species is in fact *P. depressa*. This view is consistent with the previously reported southern limit of *P. vulgata* being Odeceixe, Portugal (Fischer-Piette and Gaillard 1959; Purchon 1968) Lagos, Portugal (Hawkins pers. comm.), whilst *P. depressa* has been reported in the Western Mediterranean (Côrte-Real 1992). The situation is further complicated by Pilsbry's classification (1891) in which *P. depressa* was included as a

subspecies of *P. vulgata*. The use of the same name to describe different species and different names to describe the same species is the source of much of the confusion in patellid taxonomy. Christiaens (1973) gives a good summary of the use and abuse of synonyms. *P. depressa* has so far been recorded to penetrate the West Mediterranean basin up to Nerja (Spain) (Grandfils and Vega 1982) and in Morocco (Fischer-Piette 1935).

P. caerulea is recorded by Davies (1969a) and Bannister (1975) as one of the two *Patella* species present in the Mediterranean, the other species being *P. rustica* (= *P. lusitanica*). It consists of two populations; one in the mesolittoral zone and one in the infralittoral zone. Work in Israel records two co-existing species of *Patella* - *P. caerulea* and *P. aspera* (Lavie *et al.* 1987). These two species have also been recorded in Italy (Bacci and Sella 1970; Sella 1976; Sella and Bacci 1971). Initially work suggested that the *P. caerulea* may be a group of species and that *P. aspera* may be an ecotype of *P. caerulea* (Bacci and Sella 1970). Bowman (1981) proposed a similar relationship suggesting that *P. caerulea* may be a form of *P. aspera* associated with a particular diet and temperature regime. Further work in Italy confirmed the '*caerulea*' species complex to be made up of *P. caerulea* and *P. aspera* (Badino and Sella 1980a, 1980b).

With *Patella ferruginea* and *Patella nigra* also found it is generally accepted to be six patellid species in the Mediterranean (*P. aspera*, *P. caerulea*, *P. depressa*, *P. ferruginea*, *P. rustica* and *P. nigra*).

1.2 - Morphometric analysis

Traditionally limpet morphometrics has been analysed in one of four ways :

1 - Univariately, using just one character to give information on population structure; shell length is commonly used (Baxter 1982).

2 - Bivariately, taking this a stage further by using the combination of two or more shell characters often as a ratio or percentage (Bannister 1975; Beaumont and Wei 1991).

3 - Using shell length and the radula length as a ratio (R/C fraction = La longueur de la **R**adula : La longueur de la **C**oquille (Fischer-Piette 1934, 1935; Evans 1947). Sev-

eral authors have used model I linear regression to derive an R/C value (Bacci and Sella 1970; Christiaens 1973; Bannister 1975; Sella 1976).

4 - Using a ratio of radula length to a combination of characters, or a ratio of radula length to a measure of shell volume (Fischer-Piette 1948).

The appropriate method for any analysis is dependent upon the question it is attempting to answer. 'How does length A vary with length B in different situations?' may be answered using a ratio. But if the question 'How does size and shape vary in different situations?' is asked then it is inadvisable to use regression analysis and ratios (Sundberg 1988).

There are a number of reasons for treating ratios with caution (Atchley *et al.* 1976; Atchley and Anderson 1978).

1 - Where the degree of correlation between numerator and denominator is not very high, the resultant ratio can vary greatly dependent upon the sample it is based upon (Sundberg 1988).

2 - The ratio depends upon the denominator (size) and is hence correlated with size, unless the relationship between the numerator and denominator is linear and passes through the origin (Gould 1966; Thorpe 1983).

The majority of linear regressions used in limpet morphology to derive R/C ratios are Model I (Christiaens 1973; Sella 1976; Côrte-Real 1992). One of the assumptions of model I regression is that the operator controls the independent variable. For a definition of whether the independent variable is controlled or not see Berkson (1950). Limpet shell length is not a controlled variable and so model II regression should be used (Ricker 1973, 1975) as a model I regression coefficient "is expected to be lower in absolute value than the true slope of the functional relationship" (Sokal and Rohlf 1969). Laws and Archie (1981) suggest that for morphometric analysis model II regression is needed (citing Gould 1966; Gould and Woodruff 1978; Kuhry and Marcus 1977).

Aside from the statistical reservations, there are conceptual reasons to balk at the use of Model I regression to investigate size and shape differences:

1 - There is a high degree of variation in the shell shape and in the relationship between radula length and size of limpets. Regression analysis effectively 'hides' this variance, which is intrinsic to the morphology and is of biological importance, so should be investigated together with the underlying patterns of isometric variation.

2 - The R/C fraction uses shell length as a measure of size. Sundberg (1988) questions whether size can be represented by a single measure of distance stating that 'size should rather be viewed as a linear combination of all traits that accounts for as much as possible of the association among other distance characters'.

The limitations of the above methods and the increased access to computers has allowed a new approach to the study of molluscan morphometrics, the use of multivariate statistics. Multivariate techniques have now been used to study a variety of molluscs, for example littorinids(Janson and Sundberg 1983; Janson 1987; Sundberg 1988; Grahame and Mill 1989, 1992; Johannesson 1990) ; mussels (Vario et al. 1988; Mc Donald 1991) limpets (Corte-Real, 1992; Hernandez-Dorta, 1992). Multivariate morphometrics is a more holistic approach to variation in size and shape. A number of shell measures, when taken together, better describes shape and size differences within and between groups. Multivariate techniques can allow the visualisation of complex relationships between variables in one or two dimensional space.

R/C values are still useful as they allow for the comparison of results with those of previous authors. Indeed, provided a wide range of shell lengths are used and the limitations of the technique are acknowledged species-specific differences can be shown.

1.3 - Biochemical Genetics of *Patella*.

Bearing in mind the high morphological variation within species reported for patellids (Russel 1907; Orton 1928b, 1929; Moore 1934; Comfort 1946; Ebling *et al.* 1962; Davies 1969a; Bacci and Sella 1970; Sella 1976; Gaffney 1980; Bowman 1981), morphology alone is obviously of limited use in elucidating the taxonomy of this group.

The relatively recent emergence of enzyme electrophoresis techniques as a taxonomic tool (Nei 1972; Avise 1974; Ayala 1976, 1983; Thorpe 1979, 1982, 1983), has cast consid-

erable doubt on the validity of the specific assignments of past work (Gaffney 1980; Sella 1980). Previous studies have been largely based on morphological characters, which show a high degree of intraspecific variation. Additionally, morphological characters considered in isolation, have in the past been used in fairly limited ways, which vary between works and have recently been the subject of much criticism (see review by Sundberg 1988).

The confused, involved and often contradictory literature on the taxonomy of patellid limpets would benefit considerably from the application of biochemical genetics. This allows the possibility of individuals belonging to any particular gene pool, and hence to any particular species, to be ascertained to a very high level of statistical probability (Nei 1972; Thorpe 1979).

The most common use of enzyme electrophoresis on a taxonomic level has been to distinguish between sympatric, cryptic species (e.g. *Drosophila* - Ayala & Powell, 1972; Ayala et al. 1974; Ayala, 1975 ; sponges - Solé-Cava and Thorpe 1987; Solé-Cava *et al.* 1991 ; barnacles - Hedgecock 1979; Dando and Southward 1980 ; littorinids - Furman 1990; Ward 1990 and limpets - Murphy 1978) and to unite morphologically differentiated sympatric ecotypes (Johannesson and Johannesson 1990; Beaumont and Wei 1991; Côrte-Real *et al.* 1992).

The use of biochemical genetics in the study of taxonomy of patellid limpets has been somewhat limited to date. Hatch (1977) showed that transitional forms of *Patella*, supposedly *P. vulgata*/*P. depressa* hybrids (Fischer-Piette 1935; Evans 1953; Dodd 1957) were taxonomically synonymous with *P. vulgata*. Gaffney (1980) showed that the three species of British *Patella* (i.e. *P. aspera*, *P. depressa* and *P. vulgata*) were genetically distinct at five out of the seven loci looked at and that morphologically transitional forms between the three species were again taxonomically synonymous with *P. vulgata*. The existence of intermediate types had been used as the basis for a number of early species origin hypotheses for *Patella*. Intermediate forms were supposedly the result of incomplete speciation from a polymorphic root stock (Fischer-Piette 1935; Huxley 1942; Evans 1953), or the result of hybridisation among three previously differentiated types (Fischer-Piette 1935; Orton 1946; Evans 1958). The work of Hatch (1977) and Gaffney (1980) showed there to be no genetic basis for these hypotheses.

Badino & Sella (1980a; 1980b) looked at variation in the *Pgi* locus in three Mediterranean species: *P. aspera*, *P. coerulea* (= *P. caerulea*), and *P. lusitanica* (= *P. rustica*) and showed the locus to be diagnostic for *P. rustica*. The allozymes for this species exhibited a more anodal general zone of mobility. *Pgi* can be used as a species-diagnostic character with 99.9% probability of correctly distinguishing *P. rustica* from the other two species (Sella and Badino 1982). Later work (Sella *et al.* 1989) over 12 loci showed *Got* to be diagnostic for *P. aspera* and three loci were diagnostic for the three species (*Me*, *Mpi* and *Pgm2*).

Another use of biochemical genetics is to look at the population genetics of a species (Johnson 1974; Ayala *et al.* 1975; Ahmad *et al.* 1977; Beaumont 1982; Johnson and Black 1982; Berger 1983; Buroker 1983; Johnson and Black 1984a; Furman 1990; Johannesson and Johannesson 1990; Sara *et al.* 1990). The study of intraspecific genetic variation for both sympatric and allopatric populations can give insights into the structuring forces, and resulting structure, of a population. The investigation of allopatric populations can give measures of gene flow between populations and clues to the levels of larval dispersal.

According to the niche-width variation hypothesis (Van Valen 1965), there should be a positive correlation between the niche- breadth and the level of genetic diversity. It has been suggested that the most likely general mechanism for maintaining genetic polymorphisms is spatially heterogeneous environments (Levene 1953; Bryant 1976; Hedrick *et al.* 1976; Gillespie 1978). This positive correlation between genetic and environmental variation, has been demonstrated at the karyotypic level (Nevo 1978) and at the level of enzyme variability (Powell 1971; Mc Donald and Ayala 1974; Powell and Wistrand 1978), but many authors dispute its validity (Schopf and Gooch 1971; Somero and Soule 1974; Ayala and Valentine 1979).

Wilkins (1977) investigated the genetic variability of *P. vulgata* and *P. aspera* using two enzyme systems (*Pgi* & *Pgm*). He stated that "*P. vulgata* has an obviously broader ecological niche, judged by the greater range of habitat types and localities it occupies, than *P. aspera*". On this basis it was expected that *P. vulgata* would show more genetic variability than *P. aspera* if the niche-width hypothesis held true. The reverse was in fact found. Wilkins showed that *Pgi* in *P. vulgata* was almost monoallelic (i.e.. showed very little genetic variation). He also found that *Pgi* in *P. vulgata* was more heat stable than in *P. aspera*

(advantageous to high shore animals). On the basis of these two results he suggested that natural selection acts to maintain a single ecologically advantageous phenotype resulting in the virtual absence of allelic variants at the *Pgi* loci in *P. vulgata*. Similar results have been found in some littorinid species (Berger 1973).

Later work (Lavie *et al.* 1987) looked at allozymic variation encoded by 15 gene loci in two Mediterranean *Patella* species *P. caerulea* and *P. aspera*. It was predicted that *P. caerulea* would be the more heterozygous of the two as it appeared to occupy a broader niche, being found higher on the shore. However the results appeared to indicate a negative correlation between niche-width and genetic variation, with a greater genetic variation observed in *P. aspera*. They did not agree with Wilkins (1977) that this was indicative of natural selection acting to maintain a single ecologically advantageous phenotype. They suggested that the definition of the niche-width was erroneous and that *P. aspera* has a broader niche than *P. caerulea* on the basis of exposure to strong wave disturbance. This highlights a major problem with the niche-width variation hypothesis. It is harder to define the niche than it is to measure the genetic variation (Lavie *et al.* 1987)! It seems clear that a unidimensional interpretation of a concept involving n-dimensional hyperspace is over simplistic. Defining a niche in a real situation is impossible since most of the parameters involved are either unknown or unmeasurable. The ability of an observer to measure a niche is therefore limited. Furthermore, variation within a species may represent a variable "grain" in either perception or response of a species to its environment (i.e. two species may appear to an observer to be occupying similar niches and yet are responding entirely differently). Such ideas are encapsulated in the "Environmental grain hypothesis"(Ludwig 1950; Levene 1953; Levins 1968; Selander and Kaufman 1973). As pointed out by Whitehead (1920) 'one should seek simplicity in nature, but never believe in it.'

In the work by Badino and Sella (1980a, 1980b) the electrophoretic patterns of *P. aspera* and *P. caerulea* were found to be very similar, whilst the mobility of *Pgi* allozymes was more anodal for *P. rustica*. The form of *Pgi* found in *P. rustica* was found to be more heat stable than the form in the other two species. From this, it was tentatively suggested that the results showed selective action enabling *P. rustica* to extend its vertical range further up the shore. The similarity of the *P. aspera* and *P. caerulea* results was taken to be a product of them being subjected to the same selective action as they colonise the same zone on the shore

in Lenghorn, Italy. This coexistence is possible as they do not compete with each other for food resources (Della Santina 1992). This supports work by Murphy (1976) who studied the *Lap* locus variability in some species of American acmaeid and suggested that the similar pattern of variability was due to the co-existence of the two species in the absence of competition between them. The major criticism of both Wilkins' and Badino & Sella's work is that they only studied one or two enzyme systems which could severely bias their results. The evidence of a causative relationship between genotype and environment is absent in their studies. Indeed such a relationship is very difficult to demonstrate and evidence in the whole biochemical genetic literature is sparse. Additionally correlation between genetic variability and an environmental cline does not imply a causative relationship between the two.

Sella *et al.* (1985) showed high levels of gene flow between Tyrrhenian and Adriatic populations of *P. caerulea*. Such high levels are probably facilitated by transport of larvae along the coast. Additionally, high levels of gene flow are shown between mainland Italy and Corsica (Badino *et al.* 1986) in a study based on three loci (*Pgi*, *Pgm-1* and *Mdh-1*)

Two aspects of limpet genetics inadequately covered for Mediterranean species are: the degree of gene flow over a large geographic range; and the level of gene flow between limpets at different levels of the littoral zone.

1.4 - The Mediterranean

1.4.1 - Geological History

This account of the Upper Tertiary and the Quaternary periods is taken in the main from Blanc (1968) and Pérès (1985).

The Quaternary history of the Mediterranean (See Figure 1.1) can be seen as a succession of regressions and transgressions (See section 1.4.1.1 & 1.4.1.2 for definitions). These caused current pattern reversals in the mouth of the Mediterranean. Invasive waves of boreal, temperate or subtropical faunal species were controlled by the current systems through the Straits of Gibraltar (Blanc *et al.* 1959; Pérès and Picard, 1959). The majority of littoral introductions were of southern rather than boreal species. This is because when there was a surface water inflow into the Mediterranean (which would facilitate littoral input) it was gen-

erally a warm water inflow due to the negative water balance of an interglacial period. Equally the majority of boreal species that invade the Mediterranean were from the circalittoral or below as cold water inflow into the Mediterranean was a deep water influx during a regression. Outlined below is a brief history of the Quaternary period in the Mediterranean.

1.4.1.1 - Transgression

During a typical interglacial period the Mediterranean had a warm climate and negative water balance (evaporation > precipitation rate). The warm waters of the Atlantic extended further north and penetrated the Mediterranean through the Straits of Gibraltar - compensating the water balance. During a transgression the Mediterranean had a higher temperature and salinity than during a period of glaciation. These conditions permitted the establishment and maintenance of tropical or warm temperate littoral fauna in the Mediterranean.

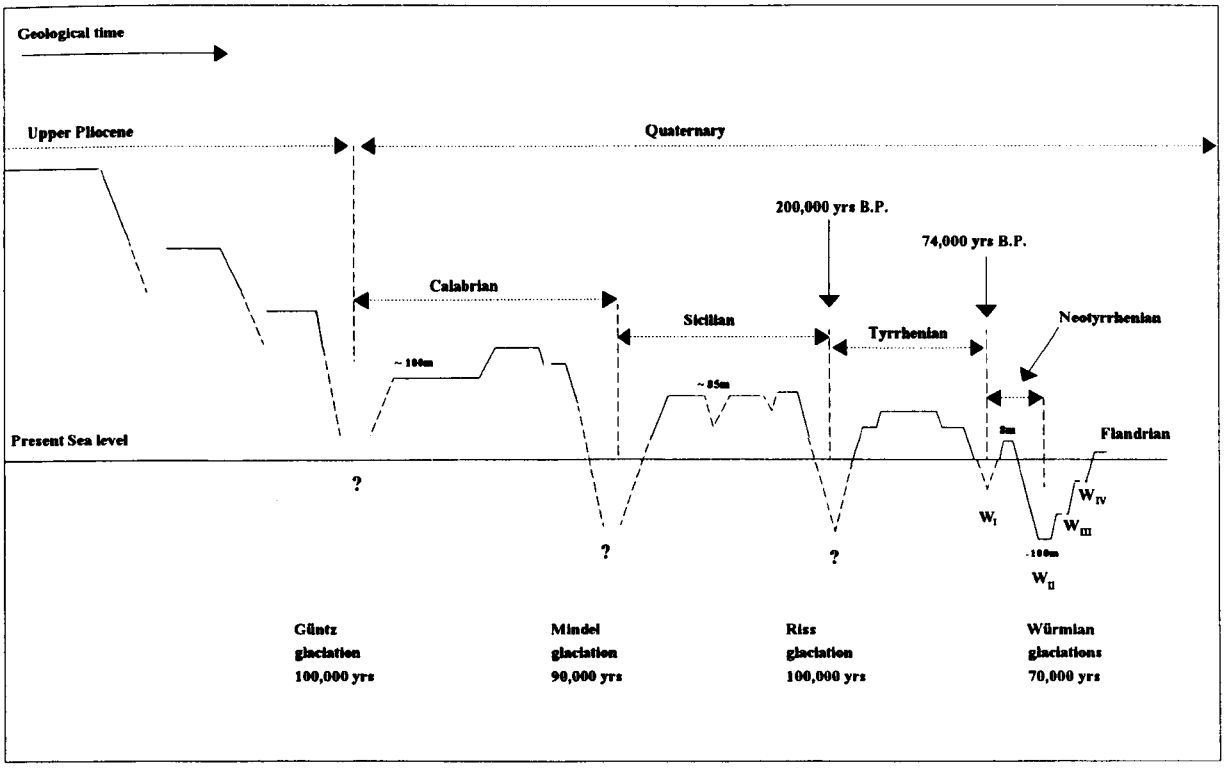


Figure 1.1 - Chronology of the Upper Pliocene and the Quarternary, showing the glacial events and the interglacial periods. The drop in sea level in the Güntz, Mindel and Riss glaciations are not known. W_I - Würmian I, W_{II} - Würmian II, W_{III} - Würmian III, W_{IV} - Würmian IV. After Blanc (1968).

1.4.1.2 - Regression

During a typical glacial period the water level in the Mediterranean fell because of eustatic regression. The Mediterranean climate became cooler and more humid, creating a positive water balance as precipitation was greater than evaporation. The surface water current reversed and flowed out of the Mediterranean while the deep water counter current flowed in. Together with a reduction in water temperature and salinity and the southerly movement of cooler Atlantic water, this allowed the introduction and maintenance of deep water and circalittoral boreal species.

1.4.1.3 - Upper Tertiary period (70- 2 my B.P.)

During most of the Tertiary the Mediterranean was not a discrete waterbody, rather it was part of the Tethys Sea, a tropical sea still connected with the Indo-Pacific. The fauna of this period was tropical and is often referred to as Paleomediterranean. During the Pliocene the region cooled and the connection to the Indo-Pacific closed. Many of the Paleomediterranean species died out. Some of those that survived may have evolved into the endemic Mediterranean fauna (Pérès 1985). However, it is very difficult to determine whether a species survived and evolved *in situ.* or whether it was extinguished in the Mediterranean and than reintroduced during one of the Quaternary transgressions.

As the Mediterranean became more temperate and cooled there was an influx of Atlantic species, many of which are still present today. There were also a few boreal species in deeper waters (e.g. *Amussium cristatum*) brought in on a deep water influx and surviving in the Pliocene.

1.4.1.4 - Quaternary (2 my B.P. - present)

1.4.1.4.1 - Calabrian period (2 - 1 my B.P.

The Calabrian period started with a weakly transgressive warm phase and was characterised by species derived from the ecological and climatic environments of the Tertiary. The lower Calabrian is marked by the Güntz glacial regression which resulted in the influx of boreal species (e.g. *Cyprina islandica*, *Pecten maximus*, *Chlamys islandicus* and *Mya trancata*) into the Mediterranean. There were few if any littoral species brought in as the water in-

flow was through deep water currents. Many species that were present in the Mediterranean during the Güntz regression, are only found in the Northern Atlantic in the present day.

Subsequently there was a second transgressive phase, stronger than the first (the Emilian) and sea levels once again rose. There was a marked reduction in the number of boreal species, coupled with an increase in temperate and subtropical species (e.g. *Pecten jacobaeus*, *Chlamys varia*, *Nassa giberrula* and *Murex trunculus* var. *conglobata*). Mars(1963) shows the presence of *Nucella lapilus*, a boreal species, and *Turris undatiruga*, a Southern species, in the same beds. This suggests that at the time conditions were in balance between climates such that some species from both could survive.

1.4.1.4.2 - Sicilian period (1 my - 200,000 years B.P.)

The Sicilian period corresponded to the interglacial period of Mindel-Riss. The lower Sicilian saw a renewed inflow of deep waters into the Mediterranean. The period was characterised by the disappearance of a large number of Paleomediterranean species. The upper Sicilian was the transgressive Palaeotyrrhenian period where the sea level was approximately 30 m above present. The geological record of this period shows the disappearance of boreal species and the appearance of a more temperate/moderately warm water fauna, as exemplified by *P. ferruginea* (Blanc 1968). The end of the Sicilian period was marked by the Rissian regression.

1.4.1.4.3 - Tyrrhenian period (200,000 - 74,000 years B.P.)

This was the Riss-Würm interglacial transgression period. The sea level during this period did not appear to extend higher than 12m above that of present. The period was characterised by the appearance of so called 'Senegalian species', these were warm, temperate species rather than true subtropical and were moved into the Mediterranean on the transgressive surface water inflow. Among those still found in the Mediterranean there are *Fissurella nubecula*, *Pirenella conica*, *Cypraea lurida* and *Thias haemastoma*.

1.4.1.4.4 - Neotyrrhenian period and Würmian regression (74,000 - 30,000 years B.P.)

There was a weak transgressive phase between stages I and II of the Würmian. The sea levels reached between 1.5 and 8.0 metres above present levels. The major regression

was Würmian II and III and included a sea level 80-100m lower than present. The paleomediterranean element is missing from the geological record of this time. This period also saw a new immigration of boreal species, which were found throughout the Western Mediterranean basin, but all are now extinct in the Mediterranean (e.g. *Buccinum undatum*, *Cyprina islandica*, *Chlamys islandica*, *Chlamys septemradiata* and *Modiolus modiolus*). The cold water fauna was much less pronounced in the Eastern Mediterranean basin, only *Chlamys septemradiata* being found. This suggests that the cooling effect in the Eastern Mediterranean basin was less pronounced then in the Western basin during the Würmian. This regressive period favouring boreal species continued into Würmian III and IV.

1.4.1.4.5 - Flandrian period (30,000 years B.P. - present)

This transgressive period corresponds to the raising of sea level to that of the present day. This transgression caused an increase in salinity and water temperature. The boreal species of the Würmian became extinct and the fauna was dominated by two elements: an Atlantic element, immigrant during the Pliocene and the endemic element developed during the Calabrian and Sicilian periods. There are relic populations from the Tyrrhenian Senegalian species immigration and the Güntz and Würmian boreal species influx. Both groups survive in localised areas where the environmental conditions are suitable.

1.4.2 - Biogeographic regions

Based on biogeography the Mediterranean can be divided into four basic regions (Pérès 1967): the Alboran Sea, the Western Basin, the Adriatic Sea and the Eastern Basin (See Figure 1.2). The present day surface current patterns show the Atlantic inflow in the surface layer (See Figure 1.3). This allows the establishment of Atlantic species in the Mediterranean. Of note is the current running along the North African coast which allows greater extension of warm- temperate Atlantic species along this coast than along the Spanish coast.

1.4.2.1 - Alboran Sea

This area at the mouth of the Mediterranean is characterised by the absence of several endemic Mediterranean species (e.g. *Antedor mediterranea* & *Eunicella cavolinii*). There are a number of Atlantic species found in the Alboran sea which are not present in other parts of the Mediterranean, most importantly for this study *P. depressa*.

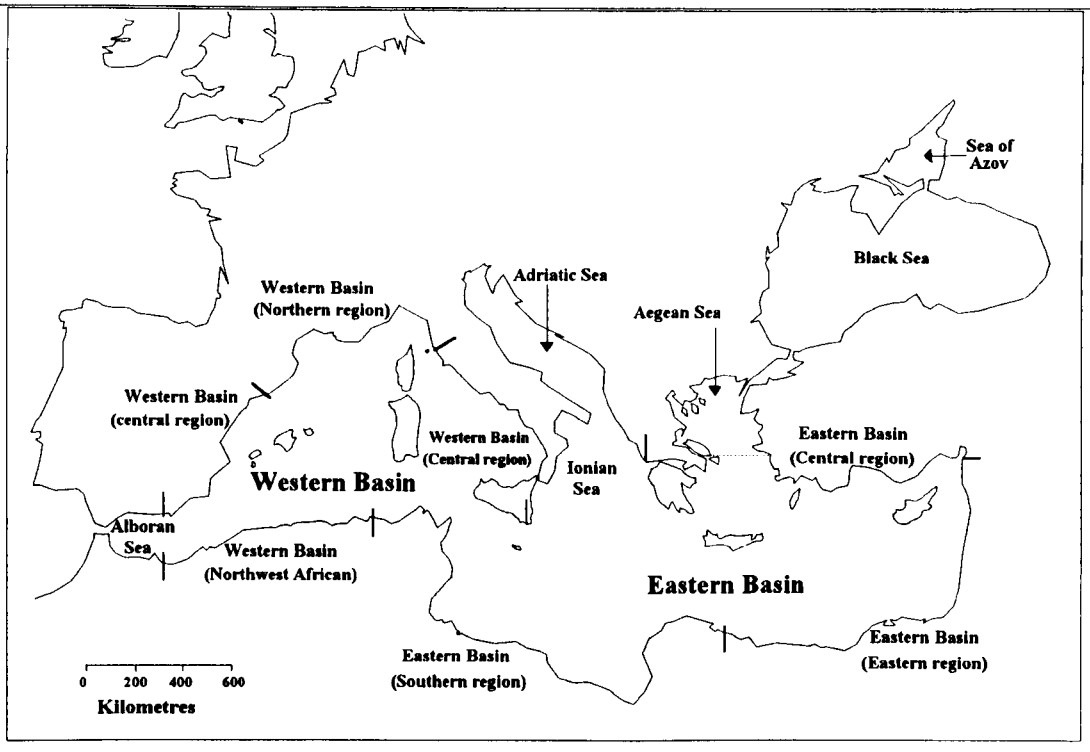


Figure 1.2 - The regions of the Mediterranean from Pérès (1967). The Mediterranean is divided up into: the Alboran Sea, Western Basin, Adriatic Sea and Eastern Basin.

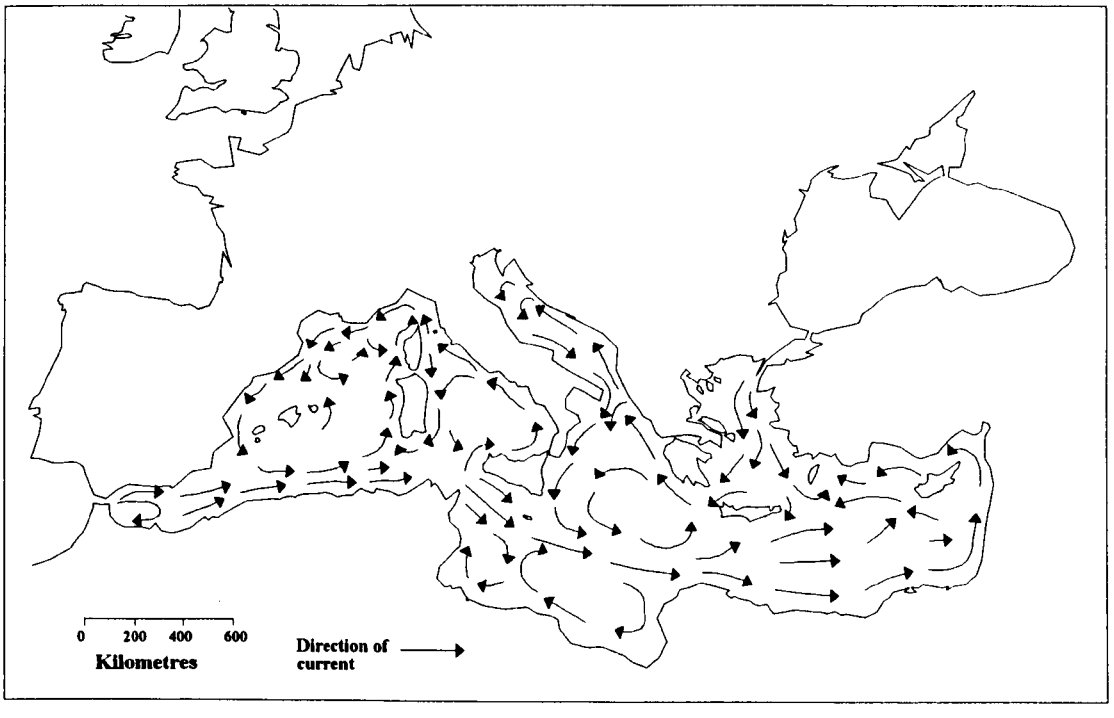


Figure 1.3 - Summer surface current patterns taken from Viale (1985). Note the extended flow along the North African coast of the Mediterranean.

1.4.2.2 - Western Basin

This area can be further subdivided into: North-west African coastal region, Central region and Northern region (Pérès 1967).

1.4.2.2.1 - Northwest African coastal region

This region contains most of the typical Mediterranean species but additionally a number of species of North Atlantic and Senegalian origin. The present inflow of Atlantic surface waters assists in the intrusion of these species. The slower modification of hydrological conditions along the coast of North Africa than along the coast of Spain increases the penetration of species along the North African coast (Pérès 1967). Thus the Senegalian species *P. safiana* extends to the coasts of Oran, but only into the Alboran sea along the Spanish coast.

1.4.2.2.2 - Central region

Additional to the areas marked (Fig 1.2) this area includes the Balearic Islands, Corsica and Sardinia. This central region has the fauna most indicative of the Mediterranean (Pérès 1967). Most endemic and Atlanto-Mediterranean species are present together with some subtropical species. The subtropical species are rare but are able to live at high densities in localised areas of higher temperature.

1.4.2.2.3 - Northern region

The fauna and flora are less diverse in this area than in the central region, due to winter cooling. The cooler conditions together with the lack of direct Atlantic inflow into this region may explain the absence of the subtropical species *P. ferruginea* and *P. safiana*.

1.4.2.3 - Adriatic Sea

Divided into three regions by Pérès (1967) this region is characterised by a number of endemic species (e.g. *Fucus virsoides*) and at the northern end it has tides larger than those in most of the Mediterranean.

1.4.2.4 - Eastern Basin

This basin can be divided into four areas: Southern region, Eastern region, Central region and the Aegean Sea.

1.4.2.4.1 - Southern region

The most subtropical region in the Mediterranean. Important populations of warm water species of Senegalian or Indo-pacific origin, maybe relic species, are found here such as *Albunea carabus* and *Ocypode cursor* (Holthuis and Gottlieb 1958)

1.4.2.4.2 - Eastern region

This area is in flux due to the immigration of Red Sea species through the Suez canal (see for examples Holthuis and Gottlieb 1958; Gorgy 1966)

1.4.2.4.3 - Central region

Several Western species are rare or absent (*Eunicella graminea*, *E.cavolinii*, *Corallium rubrum*). Warm-water species are numerous, and will probably increase due to Red Sea input. There are endemic species which are limited to this area (Pérès 1984).

1.4.2.4.4 - Aegean Sea

Fauna and flora very similar to the northern region of the Western Basin.

1.4.3 - Vertical zonation

The tidal amplitude in the Mediterranean is generally low, therefore in most localities wave surge is of greater importance in determining biotic zonation in the littoral zone. The combination of small tides and high summer rock temperatures results in a compressed but clearly delineated vertical zonation on rocky shores.

To encompass these differences, the terminology for littoral zones used in the Mediterranean is different to that for the Northern Atlantic. This study will use the zonation terms from Pérès (1967) which are based on the biotic distributions. The Italian term 'mesolittoral' will be used in preference to 'mediolittoral' due to its common usage in Mediterranean patel-

lid biology (Sella 1976) and to avoid confusion when comparing my work with the aforementioned works.

The four pertinent littoral zones are: supralittoral, mesolittoral, infralittoral fringe and infralittoral.

1.4.3.1 - Supralittoral

This is the upper littoral zone, the vertical range of which varies depending upon the degree of exposure. This zone is characterised by permanent or almost permanent emersion, with the majority of surface wetting coming from spray and waves. Where the tidal range is small this zone can be 30-50 cm in sheltered areas and 3-4 metres in exposed areas (Pérès 1967). The flora includes the blackish lichen *Verrucaria symbalana* and the unicellular algae, Cyanophyceae and Chlorophyceae. The algae occur equally in endolithic and epilithic forms (Della Santina *et al.* 1992). The fauna is dominated by *Littorina neritoides* and the isopod *Ligia italica*. *Chthamalus depressa* is found in the lower supralittoral. During storms, when the zone is well washed by waves *P. rustica* can move up into this zone to graze (Della Santina and Chelazzi 1991).

1.4.3.2 - Mesolittoral zone

The mesolittoral zone includes all the biota which are more or less regularly exposed and submerged (Pérès 1967). The upper limit of the mesolittoral corresponds to the highest level of submergence by waves or by rising of a calm sea. The lower limit corresponds to the lower level of normal emergence. The mesolittoral is commonly divided into two subzones: the upper mesolittoral where wetting is due mainly to waves and where submergence is rare and the lower mesolittoral, where submergence is more frequent and sometimes extends for prolonged periods.

1.4.3.2.1 - Upper mesolittoral

The upper limit of this zone varies between 0.5 and 2.5m above mean sea level dependant upon exposure of the site. The dominant flora are members of the Cyanophyceae (Della Santina *et al.* 1992) approximately 75% of which are epilithic. Another common species is

the brown alga *Mesopora mediterranea*. In exposed areas there is a seasonal development of a Rhodophyceae belt consisting of a turf of several interacting species.

The common fauna include *Chthamalus stellatus*, whose abundance increases in exposed areas. *Littorina neritoides* and *Ligia italica* extend down into this zone in calm conditions. This is the zone which *P. rustica* is most prevalent, though where sea conditions permit it can also be found grazing in the supralittoral and the lower mesolittoral.

1.4.3.2.2 - Lower mesolittoral

The lower limit of this zone is a few centimetres above mean sea level. Cyanophyceae, Chlorophyceae, Phaeophyceae and Rhodophyceae are all found in this zone, the majority of species being epilithic (Della Santina *et al.* 1992). As with the flora, the fauna found in this level is much more diverse than in the upper mesolittoral or supralittoral. The species composition varies with geographic location, degree of exposure and substratum type (Pérès 1967). It is generally characterised by the presence of one or more species of encrusting calcareous algae. This is the zone in which *P. caerulea* is most abundant though both *P. aspera* and *P. rustica* are also present.

1.4.3.3 - Infralittoral fringe

This zone is a compressed zone around mean sea level, its vertical extent dependant upon the degree of exposure. The algae found are predominantly Rhodophyceae forming a red algal turf. This is often dense and well developed. The predominant limpet species is *P. aspera* which is often covered in epiphytic red algae. The adults are normally found in deep depressions in the rock surface caused by decalcification of the rock.

1.4.3.4 - Infralittoral

The upper limit of this zone is where the substrata is permanently immersed. The lower level is the lowest extent of photophilic algae, which in places may be down to 40m. "The most important feature of the zone is the presence of luxuriant vegetation including many photophilous seaweeds and eelgrass (Pérès 1967). The biotic assemblage present in any area is dependant upon the geographic location, degree of exposure and type of substrate

(Pérès 1967). It is in this zone that large individuals of *P. caerulea* are found and occasionally *P. aspera* in exposed areas (Sella and Bacci 1971; Sella 1976).

1.5 - Descriptions of species studied.

1.5.1 - *P. caerulea*

Naming authority : Linnaeus, 1758

Synonyms of *Patella caerulea* (From Christiaens 1973.)

Patella alba, Da Costa, 1771

Patella crenata, Gmelin, 1791

Patella margaritacea, Gmelin, 1791 (name Chemnitz)

Patella plumbea, Röding, 1798

Patella silicina, Röding, 1798

Patella angulata, Renier, 1804

Patella squama, Blainville, 1825

Patella grisea, Blainville, (name Gmel.), 1825

Patella scutellaris, Blainville, (name Lam.), 1825

Patella grisea, Risso, 1826

Patella lugubris, Risso, (name Gmel.), 1826

Patella fragilis, Philippi, 1836

Patella subplana, Potiez & Michaud, 1838

Patella cerulea, Maravigna, 1838

Patella alba, Anton, 1839

Patella riparia, Chier., Nardo, 1847

Patella scutellaris, Lam., Reeve, 1854

Patella rubra, Pouchet, 1868

Patella caerulea var. *nacrina*, De Gregorio, 1884

Patella caerulea var. *intermedia*, *adspersa* & *stellata*, Bucquoy, Dautzenberg & Dolfus, 1886

Patella hellespontiana, Monterosato, 1888

Patella scutellina, Locard, 1891

Patella caerulea var. *major*, Pallary, 1913

Patella caliculus, Li C. Chang, 1930

Patella caerulea with var. *pyramidata*, *viridis*, *cinerea*, *pentagonalis*, *octogona*, *superposita*, Salio ms., Coen, 1933

1.5.1.1 - Description

The original description by Linnaeus (1758) is a short one "testa subangulata, striis numerosis inaequalibus, subtus caerulea. Habitat in M. Mediterraneo"

Patella caerulea is generally accepted as the blue limpet of the Mediterranean (Christiaens 1973). It is one of the most variable species with respect to the coloration of the soft-body parts and shell shape, form and coloration (Sella 1976).

1.5.1.1.1 - Soft body parts

The foot colour is very variable ranging from dark grey through to orange/beige. Often it is not of uniform colour, but the base will have a grey rim and a lighter coloured centre. The head tentacles vary between grey and translucent yellow/orange. The pallial tentacles are generally white, although they vary greatly. The colour varies from stark white to orange, the orange coloration is particularly prevalent in infralittoral individuals, which often have an orange base to the tentacles. In some individuals the tentacles are clearly visible and relatively broad, whilst others have very fine, barely visible tentacles.

1.5.1.1.2 - Shell characteristics

As with other species of this genus a large number of subspecies have been described based primarily on shell form. Pilsbry (1891) considered there to be 6 forms :

1 - *fragilis* Phil. - shell thin, the radiating striae very fine.
2 - *intermedia* Bucq., Dautz. & Doll. - intermediate between regularly oval and the polygonal forms.
3 - *adspersa* Bucq., Dautz. & Doll. - dotted with white on a greenish-grey background.
4 - *subplana* Pot. & Mich. - large, thin, pentagonal, the apex quite anterior.
5 - *tarentina* von Sal. - conspicuously rayed with brown; nearly smooth.
6 - *spinulosa* Bucq., Dautz. & Doll. - ribs spinose.

var. *crenata* d'Orb. - depressed, irregularly oval, with numerous rather low riblets, covered in small granules, yellowish-brown or tawny outside; inside usually bluish and iridescent, white in the middle. Found in the Azores and the Canaries.
var. *lowei* d'Orb - depressed, spreading, angularly ovate, solid, the surface densely ribbed, ribs unequal, scaly especially towards the margins; colour varying from dull brown or rust red to blackish-brown, sometimes rayed with white; inside varying from white to deep blue, irridescent, the central area usually white and much thickened, callous. Found in Madeira, Azores, Cap Verde and Canaries.

This variety is now generally accepted to be a separate species endemic to the Atlantic islands (see Côrte-Real 1992).

Christiaens (1973) mentions 11 varieties :

1 - *plana*

2 - *pyramidata* Coen.

3 - *imitans* Monts.

4 - *centragonalis* Coen.

5 - *subplana* Pot. & Mich.

6 - *scutellaris* Blainv.

7 - *pentagonalis* Coen.

8 - *stellata* Bucq., Dautz. & Doll.

9 - *adspera* Bucq., Dautz. & Doll.

10 - *fragilis* Phil.

11 - *specialis* Monts.

Clearly a single description is of little use in a situation where there is so much variation. In an attempt to enable adequate description of patellids and to investigate variation, seven pairs of mutually exclusive characters have been used (Bacci and Sella 1970; Sella and Bacci 1971). As a technique for describing individuals this approach is very useful. Care must be taken, however, as some of characters are negative (ie the absence of a trait) and some are not truly exclusive and open to misinterpretation.

1.5.1.2 - Radula

Powell (1973) gives the radula formula as 3+1+(2+X+2)+1+3, (with the **X** representing a narrow vestigial rachidian plate). The four functional centrals (unicuspid teeth) are in a straight row.

Fischer-Piette (1935) gives an R/C (Radula length/Shell length) value of 1.1 with extremes of 0.75 & 1.5 for individuals from the Algerian/Moroccan coast. From the Spanish coast R/C values for Malaga were 1.3 (range 1.1 - 1.6). In those taken from the North African coast the pluricuspid tooth is characterised by three unequal sized cusps; the internal is the smallest and the median the largest; the median and external lie parallel to one another; the two sides of the median cusp are approximately equal in length (see Fischer-Piette 1935). *P. caerulea* from the northern Mediterranean coast around Spain has pluricuspid teeth noticeably different in form and similarities with *P. depressa* were noted (Fischer-Piette 1935).

Individuals collected from the French Mediterranean coast (Evans 1953) were described as similar to *P. aspera*, the main difference between the two species being the relative size of the two major cusps. In *P. aspera* the external cusp is taller and broader than the central cusp. In *P. caerulea* the central cusp is equal in size or larger (see Evans 1953). It is possible that these specimens were in fact mis-identified *P. aspera*, as the presence of this species in the Mediterranean was not widely known.

The unicuspid teeth in *P. caerulea* show a wide variation in form (Fischer-Piette and Gaillard 1959). There is, however, a species-specific feature: the two longitudinal sides of the dorsal band lie almost parallel to each other. This feature is fairly subjective so is not the best method of differentiating between species.

1.5.1.3 - Distribution

P. caerulea is found throughout the Mediterranean. Fischer-Piette and Gaillard (1959) identified the western limit as Tangiers, Morocco. It is found all the way to the eastern end of the Mediterranean, in Israel (Lavie *et al.* 1987). Talmadge (1971) reported finding *P. caerulea* at Zanzibar. As this is on the east African coast, the report needs confirmation

and is highly suspect. Evans reports the species on the Basque coast (Evans 1953), this again is unlikely and has not been confirmed subsequently.

1.5.2 - P. aspera Röding, 1798 *or P. ulyssiponensis* Gmelin, 1791

Synonyms of *Patella ulyssiponensis*, from Christiaens, 1973

Patella auricula, Da Costa, 1771

Patella depressa, Pennant, 1777

Patella langula, Meuschen, 1778

Patella spinosula, Meuschen, 1787

Patella ulyssiponensis, Gmelin, 1791

Patella repanda, Gmelin, 1791

Patella angulosa, Gmelin, 1791

Patella vulgata, J.B., Fisscher L., (name Lam.), 1791

Patella tarentina, von Salis, 1793

Patella aspera, Röding, 1798

Patella aspera, Lamarck, 1819

Patella tarentina, Lamarck, 1819

Patella bonardii, Payraudeau, 1826

Patella vulgata ß *costata* (= *P. albicosta* Marks), Forbes, 1838

Patella donacina, Schroeter, Anton, 1839

Patella lowei d'Orbigny, 1840

Patella tarentina, Delessert, 1841

Patella athletica, Bean, 1844

Patella vulgata L. var. *albumena*, Brown, 1844

Patella tarentina, Lam. with var. *elatior* and *costulata*, Middendorf, 1849

Patella azorica, Nuttal, Jay, 1852

Patella spinulosa Meusch., Mörch, 1852

Patella ulyssiponensis var. *major*, Mörch, 1852

Patella spectabilis, Dunker, 1853

Patella scutellaris Lam. var. ß *depressa* Phil., Danilo & Sandri, 1856

Patella baudonii, Drouet, 1857

Patella vulgata L. var. *depressa* & *caerulea*, Jeffreys, 1865; 1869

1.5.2.1 - Description

Naming priority for this species should go to Gmelin 1791 as *Patella ulyssiponensis* (Christiaens 1973). However, as the vast majority of literature refers to it as *P. aspera* Röding (1798) or *P. aspera* Lamarck (1819) it shall for the sake of clarity be referred to as *P. aspera* Röding in this study.

Forbes & Hanley (1853) give a good description of a *Patella athletica* Bean (= *P. aspera*) from British waters "shell rather depressed ranging from rounded, ova, ovate to elliptical. The colour is invariably much lighter than that of *P. vulgata* and is either whitish or of a pure orange-yellow. The mantle is edged with flaky-white jointed filaments of only two lengths, twice as short and twice as thick as *vulgata*'s. The foot is various hues of orange-yellow". Eslick (1940) gives the following description of *P. depressa* (= *P. aspera*) from the Isle of Man: "the foot varies from a slightly pinkish-cream through creamy-peach and orange peach to a brilliant and full orange. The outline of the shell is elongated and tapered towards the anterior end to which the apex often approximates itself. The nacre of the shell is always a rich milky white". The very wide variation in foot colouration is obvious from this description.

Christiaens (1973) using the name *Patella ulyssiponensis* Gmelin breaks the species down into five subspecies:

1 - *Patella ulyssiponensis bonardii* Payraudeau - This is the form most common in the Mediterranean, especially in Corsica. A type specimen is kept in the Paris museum, in the 1824 Payraudeau collection.

2 - *Patella ulyssiponensis athletica* Bean - conical shell with large prominent rays. It is common in North, West and Southern England with the Isle of Wight as its southern limit (Purchon 1968); present along the French coast at I'île de Saint-Marcouf (Fischer-Piette 1941); Norway between Stavanger and Bergen (Kolstad 1959) and it is rare in Spain.

3 - *Patella ulyssiponensis pontica* Monterosato - Found in the Black Sea : Russian coast (Iljina 1966) and Bulgarian coast (Kaneva 1960). Mentioned by Bâcescu, Müller & Gomoiu, (1971) in Romania where it is very rare or not present. Samples of these popu-

lations would be desirable, in order to compare their genetics with the Mediterranean species, in order to verify their identification.

4 - *Patella ulyssiponensis aspera* Röding - The sub-species principally found in the islands off west Africa. The typical form is *depressa*, coloured, subpentagonal with numerous regular ribs. It is also found in Madeira where the vernacular name is 'lapa branca' and in the Azores where it is called 'lapa brava'. Its southern limit is reported as Angola and St Helena, but requires confirmation.

5 - *Patella ulyssiponensis deserta* - Only one individual collected, in the deserted islands in Madeira, characterized by an elongated shell and shiny white interior with a thin brown border. The foot is a peach colour as with other *Patella ulyssiponensis*. It has an unusual unicuspid tooth structure. Again further specimens for genetic assay would be desirable to verify the identification of this entity as a variety of *P. aspera*.

1.5.2.2 - Radula

Fischer-Piette (1941) gives the R/C value for *P. aspera* from Northern France as 1.15 (range 0.95 - 1.40). Of the three cusps of the pluricuspid tooth, the internal one is the smallest. The median and external cusps are sub-equal, with the external cusp dominating. These cusps do not lie parallel but converge distally (see Fischer-Piette 1935). Evans (1947) confirmed the structure of the three cusps of the pluricuspid tooth, showed further variation around this form and included in the description a fourth cusp. This cusp lies outside the main external cusp (see Evans 1947).

Describing the unicuspid tooth Fischer-Piette & Gaillard (1959) found little variation between sites on either side of the mouth of the Mediterranean (South Portugal and Morocco). The tooth has the crochet deeply inserted in the basal part by means of an angular line (see Fischer-Piette and Gaillard 1959).

1.5.2.3 - Distribution

Found throughout the Mediterranean, including the eastern basin and Israel (Lavie *et al.* 1987). Present along the western coast of Europe with a Northern limit in Norway between Stavanger and Bergen (Kolstad 1959). The Southern limit is unknown, but it is present as far south as Angola and St Helena (Christiaens 1973).

1.5.3 - P. rustica

Naming authority : Linnaeus, 1758

Synonyms of *Patella rustica*, from Christiaens, 1973

Patella granularis L., Schroeter, 1784a
Patella lusitanica, Gmelin, 1791
Patella squamata, Röding, 1798
Patella puncatata, Lamarck, 1819
Patella subgranularis, Blainville, 1825b
Patella variabilis, Risso, 1826
Patella olivacea, Anton, 1839
Patella nigropunctata, Reeve, 1854
Patella lusitanica var. *minor*, Marion, 1883
Patellastra lusitanica Gmel., Monterosato, 1884
Patellastra lusitanica Gmel., Thiele, 1891
Patella rusitca L. with var. *major* and *maroccana*, Pallary, 1938
Patella lusitanica Gmel. with var. *irregularis* Mont. ms. and *spadicea* , Coen, 1933
Patella lusitanica Gmel. var. *orientalis*, Pallary, 1938
Patella lusitanica Gmel., Fischer-Piette and Gaillard, 1959

1.5.3.1 - Description

The foot varies in colour from orange-beige, through to dark grey. The pallial tentacles are diagnostic in that they are transparent, unique in Mediterranean species and a character only *P. vulgata* shares of the patellids in the North-east Atlantic.

A clear description was given by Dillwyn (1817); shell conical, very entire, with 50 narrow , obtuse longitudinal ribs; shell height varies considerably, some shells being much elevated and others much depressed; the colour is whitish, variegated with brown or pale chestnut longitudinal rays. Sometimes rays are spotted with yellow. The inside is bluish white, more or less glossy, and the muscular impression ferruginous or tawny.

Also described by Christiaens (1973) as having a conical shell that is rounded with a subcentral apex. The sculpture being rather variable, with numerous fairly regular shiny ribs, generally granular with black nodules. The interior having 11 or 12 principal brown rays.

1.5.3.2 - Radula

The radula of this species is very distinctive. Its R/C value is much higher than other Mediterranean species: *P. lusitanica* (=*P. rustica*) from Saint-Sebastien R/C = 3.3(range 2.7 to 4.6) Fischer-Piette, (1935); *P. rustica* from the west coast of France R/C = 2.95 (range 1.95 to 4.24) Evans (1958). The other distinctive feature is a prominent rachidian tooth in a horizontal shaped radula (Powell 1973), a structure only shared by *P. ferruginea* of Mediterranean species from which it is readily distinguishable. Powell (1973) gives its radula formula as 3+1+(2+1+2)+1+3.

The pluricuspid tooth has a poorly developed external cusp and a fairly elongated median cusp (Fischer-Piette 1935). The unicuspid tooth is very variable in both the basal part and crochet shape (Fischer-Piette and Gaillard 1959). In general the crochet is inserted in the basal part of the tooth forming a slanting, sinuous line. The angle formed by the crochet and the basal part is approximately 90°.

1.5.3.3 - Distribution

Throughout the Mediterranean with the Eastern limit at Israel (Hass 1937) and Syria (Pallary 1938). Found also along the Atlantic coast of Spain, Portugal and Morocco with the northern limit at Biarritz (Purchon 1968). The southern limit is not clearly known but probably extends beyond Mauritania where Christiaens (1973) found numerous specimens.

1.5.4 - *P. depressa*

Naming authority : Pennant

Year : 1777

Synonyms of *Patella depressa*, from Christiaens, 1973

Patella auricula, Da Costa, 1771
Patella laevigata, Gmelin, 1791
Patella conus, Röding, 1798
Patella tuberculifera, Lamarck, 1819
Patella tuberculifera, Lam., Delessert, 1841
Patella vulgata var *communis*, Brown, 1844
Patella vulgata, d'Orbigny, 1849
Patella electrina, Reeve, 1854
Patella vulgata L. var. *intermedia*, Murry, 1857
Patella vulgata L. var. *intermedia* (Knapp), Jeffreys, 1865
Patella taslei, Mabille, 1888
Patella goudoti, Mabille, 1888
Patella plumbea Lam. *vatheleti* Pilsbry, 1891
Patella mabillei, Locard, 1892
Patella intermedia Jeffr. with var. *splendida* & *marteli*, Dautzenberg & Durouchoux, 1906
Patella vulgata L. var. *debilis*, Pallary, 1920
Patella intermedia Jeffr. var. *hidalgoi*, Fischer-Piette, 1953
Patella intermedia Jeffr. var *higuerensis* Fischer-Piette & Gaillard, 1959
Patella intermedia Jeffr. var *miniata*, Christiaens, 1965
Patella intermedia (Knapp) Murray with var. *pennanti*, Christiaens, 1967

The most commonly known synonym is *P. intermedia*, this name is given priority by some authors (Christiaens 1973). Tomlin (1923) gives a description of Pennant's *P. depressa* type specimen from the British Museum. This description is the same as *P. vulgata* var. *intermedia* Jeffreys, it is also without doubt the species that we call *P. depressa*. On this basis priority is given to *P. depressa* (see also Fretter and Graham 1976).

1.5.4.1 - Description

Evans (1947) provides a good description of this species : the inside of the shell is only occasionally of a golden yellow, generally it is of a darker hue and streaked with rays from the shell margin. The rays are usually very dark ranging from chocolate brown to black; they vary in width; sometimes the entire lower half of the shell is dark with lighter streaks and sometimes the background is light with dark rays. The head scar is generally of a dark colour. Its most distinctive character in the inner surface of the shell is the white marginal rays. The colour of the foot is grey-black, grey, flesh-coloured, or occasionally yellowish-cream. The mantle tentacles of *P. depressa* are white and visually distinctive.

Christiaens (1973) who gives priority to *P. intermedia* (Knapp) Murray (1857) divides the species into eleven varieties based on morphological differences:

1 var. *taslei*, Mabille, 1888 - quite abundant in Britain and Spain. A variety with quite a conical and elevated shell bearing dark, well marked interior rays, grouped into twos or threes.
2 var. *splendida*, Dautzenberg and Durouchoux, 1906 Shell interior has large black rays and black muscular impression.
3 var. *marteli*, Dautzenberg and Durouchoux, 1906 Shell has ash grey colouration, white callosite, striated with black rays.
4 var. *goudoti*, Mabille, 1888 Large shell, depressed with rays or beginnings of rays that are brown-black on a shiny yellow background.
5 var. *hidalgoi*, Fischer-Piette Shell with rounded sides,from a type specimen of *P. vulgata* from Hidalgo (1917).
6 var. *higuerensis*, Fischer-Piette and Gaillard, 1959 glossy blue-black interior with some very fine white lines, locality type Cabo Higuer, Spain.
7 var. *miniata* Christiaens vermillion colour, orange callosite, a single example found at Baie de Cadiz, Spain.
8 var. *mabillei*, Locard, 1892 Pale red exterior with light yellow to red-orange interior, found in Morocco.
9 var. *Pennanti*, Christiaens created to cover the description of the *P. depressa* type by Pennant.

10 var. *auranti*, Dautzenberg light yellow interior, no rays, yellow - orange callosite. Found notably in the South-west of France and the Atlantic coast of Morocco.
11 subsp. *vatheleti*, Pilsbry black callosite, white rays with black or brown intervals; Senegal.

var. *roberti* variety for shells from the l'île de Gorée of a uniform deep blue with interior rays that are small or indistinct.

1.5.4.2 - Radula

Fischer-Piette (1935) gives R/C value of 2.10 (range 1.6 to 2.5) for individuals from the coast of Northern France. Evans (1958) found averages of between 1.59 (le Croisii) and 1.91 (Swanage), with range of 1.04 to 2.60.

The pluricuspid tooth is characterised by three unequal cusps, the internal one being the smallest and the median the largest. The median and external cusps lie parallel to each other. The tips of the cusps are rounded in an arrow-like fashion. The median cusp is unusual, having unequal sides; the internal side is shorter than the external side. The diagrams of Evans (1947) conform with this description, though the tooth form is quite variable.

The unicuspid tooth is described by Fischer-Piette and Gaillard (1959). In most individuals examined the crochet forms an obtuse angle with the basal part. A distinctive character is the hook-like contour of the inferior half of the dorsal line

1.5.4.3 - Distribution

Found on the European Atlantic coast with a Northern limit at l'île de Saint-Marcouf, France (Fischer-Piette 1941). In Britain it is not found east of the Isle of Wight (Evans 1953) and its northern extreme is Anglesey (Crisp and Knight-Jones 1955; Lewis 1964). It is absent from the east coast of Ireland and is rare on the west according to Fretter and Graham(1976). These reports of *P. depressa* in Ireland are most unlikely and it is probably absent from Ireland altogether (Hawkins *pers. comm.*). The southern limit is Senegal (Pilsbry 1891) this being the potential subspecies *P. depressa vatheleti*. It is not present throughout the Mediterranean, but has been found in the Alboran Sea as far as Nerja on the Spanish coast (Grandfils and Vega 1982).

1.5.5 - Patella (laevipatella) nigra

Naming authority : Da Costa, 1771

Synonyms of *Patella* (*laevipatella*) *nigra*, from Christiaens, 1973

Patella squamata, Gmelin, 1791
Patella guineensis, Gmelin, 1791
Patella plumbea, Lamarck (name Röding 1798), 1819
Patella safiana Lamarck, 1819
Patella crenata = *P. nigra* Humph. and Da Costa, Dillwyn, 1823
Patella lasiana Lam., Deshayes, 1840
Patella algira, Deshayes, 1840
Patella safiana Lam., Delessert, 1841
Patella conspicua, Philippi, 1851

Patella safiana (Lamarck) is the most commonly used synonym and is still used in contemporary work (Renault 1971; Frenkiel and Moueza 1982).

1.5.5.1 - Description

Oval shell with about 45 groups of rays, each group generally comprises three ribs of which the central one is the largest. The exterior is black with dull grey in between ribs; the interior is shiny blue to silvery blue; callosite porcelain white to brown-orange, often thick with the head scar well marked; black or pale-brown border in the interior. The apex is sub-central located up to 1/3 from the front (Christiaens 1973). The entire foot is black or dark grey (Fischer-Piette 1935), the mantle is very thin with a green-yellow tint. There are large yellow pallial tentacles with numerous small tentacles of the same colour in between.

1.5.5.2 - Radula

The species has a V-shaped radula, with a clearly visible rachidian tooth. Fischer-Piette (1935) and an R/C value of around 0.86, Christiaens (1973) gives an R/C of 1.02. Both are from single individuals and thus of limited use.

The marginal teeth are of two types, with the external tooth lying below the other two. Of the four unicuspid teeth, the two external ones are larger and lie below the internal ones (Fischer-Piette 1935). The pluricuspid teeth sit below the line of the two external lateral teeth, completing the V-shape. The tooth has four cusps, two small cusps situated one on either side of two larger internal cusps (Fischer-Piette 1935). The small rachidian tooth sits in between the two internal unicuspid teeth; it has a very large basal part in comparison to its single cusp. Frenkiel and Mouëza (1982) note that the external cusp on the pluricuspid tooth is rounded, also the basal part of the external unicuspid tooth is different from the internal tooth. They suggest that these differences are sufficient to warrant retaining *P. safiana* as a Mediterranean sub-species of *P. nigra*. This has not been confirmed by other authors and would need further investigation. It is likely that *P. nigra* encompasses more than one species identity.

1.5.5.3 - Distribution

Its southern limit is not known although it has been reported around Senegal (Christiaens 1983). It is supposed to occur in Angola and Namibia (Hodgson *pers. comm.*). It extends up the African coast into the Mediterranean, being present along the coast of Morocco and West Algeria (Pallary 1900; Pallary 1920) as far as Alger (Frenkiel and Mouëza 1977; Frenkiel and Mouëza 1982). It is rare on the southern Spanish coast of the Mediterranean at Malaga (Grandfils and Vega 1984) and has been found in Estopona (Hawkins *pers. comm.*).

1.5.6 - P. ferruginea

Naming authority : Gmelin, 1791

synonyms of *Patella ferruginea*, from Christiaens, 1973

Patella gorgonica, Da Costa, 1771
Patella cypria, Gmelin, 1791
Patella ferruginea, Gmelin, 1791
Patella plicaria, Gmelin, 1791
Patella medusa, Röding, 1798
Patella pyramidata, Lamarck, 1819
Patella tuberculata Dillwyn, Sowerby, 1825
Patella stella, Risso, 1826
Patella rouxii, Payraudeau, 1826
Patella lamarckii, Payraudeau, 1826
Patella plicata, Costa O.G., 1829
Patella costoso-plicata Mart., Mörch, 1852
Patella rouxi var. *depressa*, Rigacci, 1866
Patella barbara L., Petit de la Saussaye, 1869
Patella ferruginea with var. *sitta*, *imperatoria* and form *percostata*, De Gregorio, 1884
Patella lampedusensis, De Gregorio, 1884
Patella ferruginea with var. *cometa*, *antiquorum* and *praehistorica*, Monterosato, 1888
Patella ferruginea form *stellata*, Pallary, 1900
Patella ferruginea imperatoria greg., Marcy and Bot, 1969

1.5.6.1 - Description

P. ferruginea is very distinctive, characterised by a very thick, strong, heavily ribbed shell. The 30 to 50 radial ribs are large, strongly projecting and gnarled but not granular. The shell exterior tends to be light russet or ashen in colour with concentric red-brown or rust coloured rings often more noticable in juveniles (Grandfils 1982). The interior of the

shell is a white nacre sometimes with a bluish tint, there is a black border to the internal circumference of the shell.

The foot is uniform in colour and generally beige-orange (Fischer-Piette 1935) although it can be black, blue or reddish in juveniles (Grandfils 1982). The column of the foot is blue-black, the mantle is thin and there are clearly visible and pronounced pallial tentacles.

1.5.6.2 - Radula

Powell (1973) gives the radula formula as 3+1+(2+1+2)+1+3. For individuals from Morocco Christiaens (1973) gives an R/C value of 1.83 (range 1.29 to 2.73); Fischer-Piette (1935) gives an average R/C value of 1.75. *P. ferruginea* has, in common with *P. rustica*, a horizontal tooth alignment and a visible rachidian tooth in its radula. The pluricuspid and unicuspid tooth structures are similar to *P. caerulea* (Fischer-Piette 1935; Powell 1973). The pluricuspid tooth has three major cusps, the internal cusp is the smallest and the median cusp is dominant. The rachidian tooth readily distinguishes this species from *P. caerulea* and the form of the pluricuspid tooth distinguishes it from *P. rustica*, from which it is readily distinguished by shell form.

1.5.6.3 - Distribution

P. ferruginea is largely confined to the Western Mediterranean basin, and appears to be getting increasingly rare (Fischer-Piette 1935; Christiaens 1973; Powell 1973). On the Southern coast of the Mediterranean, the Eastern limit is Oran, Algeria. The Western limit is Ceuta, Morocco (Pallary 1900; Frenkiel 1973; Frenkiel and Mouëza 1977, 1982).

1.6 - Aims of this work

This work will largely concentrate on the genetic and morphological variation of the dominant mesolittoral limpet in the Mediterranean, *P. caerulea*. This species shows very high intra-specific variation in shell and soft body characters. It is hoped to determine the degree to which this variation is environmentally or genetically controlled, between different shore zones and over the full geographic range of the species. The degree of genetic homogeneity of *P. caerulea* throughout the Mediterranean will be examined along with its degree of relation to the Atlantic species that occupies a similar zone on the shore, *P. depressa*. Finally the morphological variation of the six species of *Patella* found in the Mediterranean will be examined and distinguishing characters that transcend this variation will be outlined.

Chapter 3 will concentrate on the two morphotypes of *P. caerulea* that occur where it is bimodally distributed on the shore. It is hoped to determine whether the mesolittoral and the infralittoral morphs are genetically distinct, or two parts of a single genetic population. Further, to use multivariate statistics to determine to what degree the shell shapes of the two morphs are discrete.

The fourth chapter will examine the genetic and morphological variation of *P. caerulea* throughout its geographic range. The inferred level of gene flow will be assessed, to determine whether there is free gene flow throughout this range and to see if samples from throughout the Mediterranean can be regarded as conspecific. The morphometric work will look for regionally dictated shell shapes and examine the level of intra-specific variation in shell form.

In Chapter 5 the morphological and genetic differences between the morphologically similar species *P. caerulea* and *P. depressa* will be examined. Diagnostic genetic and morphological traits will be looked for to confirm that the two are indeed separate species and aid the differentiation of the two. The inferred levels of gene flow and genetic similarity will be used to suggest reasons for their present distributions. The final chapter will look at the variation in shell shape and soft body characters within and between all six of the *Patella* species found in the Mediterranean. The objective being to provide means of identifying the species in the field, which is essential for ecological work.

2 - General Materials and Methods

2.1 - Collection of limpets

Samples of limpets were collected from nine countries in the Mediterranean and from two sites outside the mouth of the Mediterranean (See figures 2.1 & 2.2). In each country the endemic species were collected from between one and three sites. The samples actually used in this work are summarised in Table 2.1. Where possible limpets were collected from moderately exposed breakwaters to standardize habitats between countries.

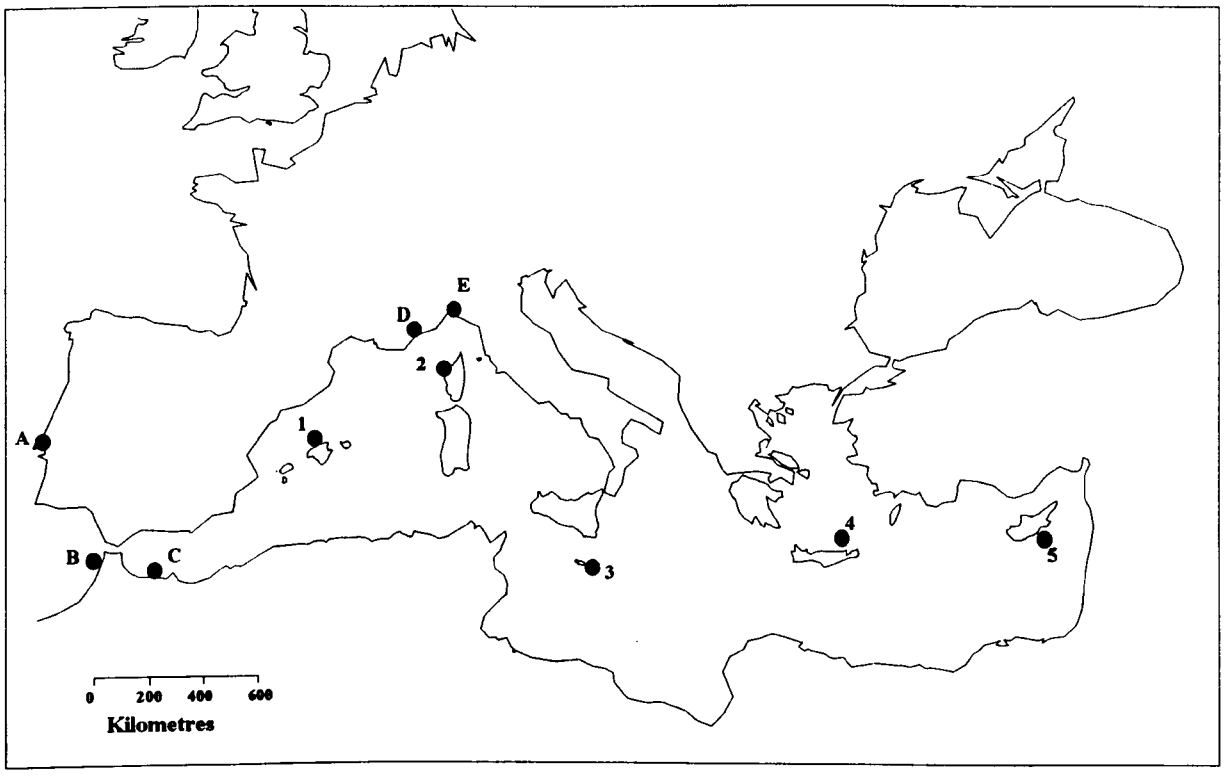


Figure 2.1 - Locations of the sampling sites in and around the Mediterranean (A=Estoril, B=Tangiers, C=Jbel Musa, D=St Tropez, E=Genoa). The numbers 1 to 5 refer to more detailed maps of the islands showing the location of the sampling sites (see figure 2.2).

Breakwaters were chosen as they provide a very wide range of microhabitats, due to the orientation of blocks and the differing materials blocks tend to be made of. This means that the limpets at every site are occupying a wide range of microhabitats hence much of the potential for phenotypic plasticity in shell form possessed by each limpet can be realized. If there are genotypes that are habitat-specific, samples from breakwaters will contain a mixture

of genotypes, each specific to a different habitat. By maximising the variation measured, at each site, it follows that any genetic or morphometric differences found between shore levels, sites or countries will not be an artifact of sampling different habitats in different countries, but will be a site/country specific difference.

Table 2.1 - Summary of the locations of the collection sites and the number of each species collected at each site. The letters in the location column refer to the maps (Figures 2.1 & 2.2) showing the location of the sites.

Country	Location	Sites	Breakwater	Species	Number collected
Portugal	A	Estoril		P. depressa	100
Morocco	B	Tangiers	✓	*P. depressa* *P. safiana*	165 66
	C	Jbel Musa		P. depressa	66
France	D	St Tropez		P. caerulea P. rustica	20 84
Italy	E	Genoa	✓	*P. caerulea*	117
Mallorca	F	El Arenal	✓	*P. caerulea*	153
	G	Mal Pas		P. caerulea	110
	H	Porto petro		*P. caerulea*	110
Corsica	I	Calvi	✓	*P. caerulea*	97
	J	Isle Rousse	✓	*P. caerulea* P. ferruginea *P. rustica*	99 15 80
Malta	K	Ghallis Point		P. aspera P. caerulea	39 100
	L	Xghajra		P. caerulea	199
Crete	M	Irepetra	✓	*P. aspera* P. caerulea	44 55
	N	Sitia	✓	*P. caerulea*	99
Cyprus	O	Limassol	✓	*P. caerulea*	80
	P	Larnaca	✓	*P. caerulea*	37

Samples of up to 100 individuals per species were then collected from the mesolittoral at each site (sometimes limited by low abundance or sometimes reduced by high mortality during transit). At the two locations where infralittoral samples were collected this was done by snorkelling and the samples were taken from between 0.5 and 6 metres below the infralit-

toral fringe.The limpets were transported, alive, in a coolbox, back to the Isle of Man. They were then transferred to a sloping tiled bench, flushed with sea-water until required.

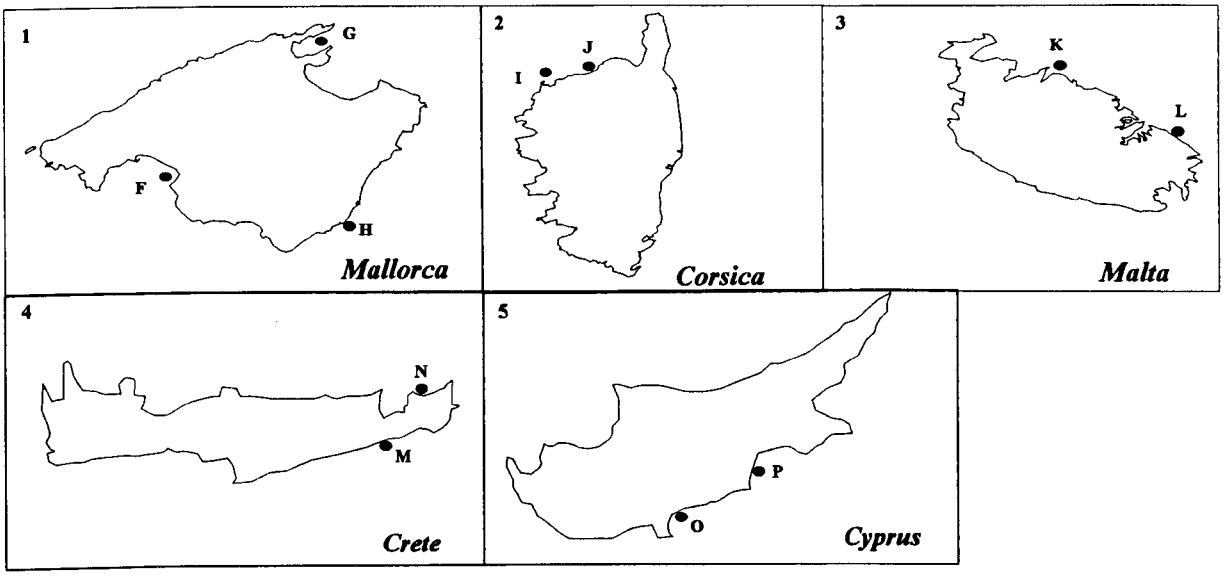


Figure 2.2 - Detail maps of the islands where samples were collected, showing the locations of the sample sites (F=El Arenal, G=Mal Pas, H=Porto Petro, I=Calvi, J=Isle Rousse, K=Ghallis Point, L=Xghajra, M=Irepetra, N=Sitia, O=Limassol, P=Larnaca).

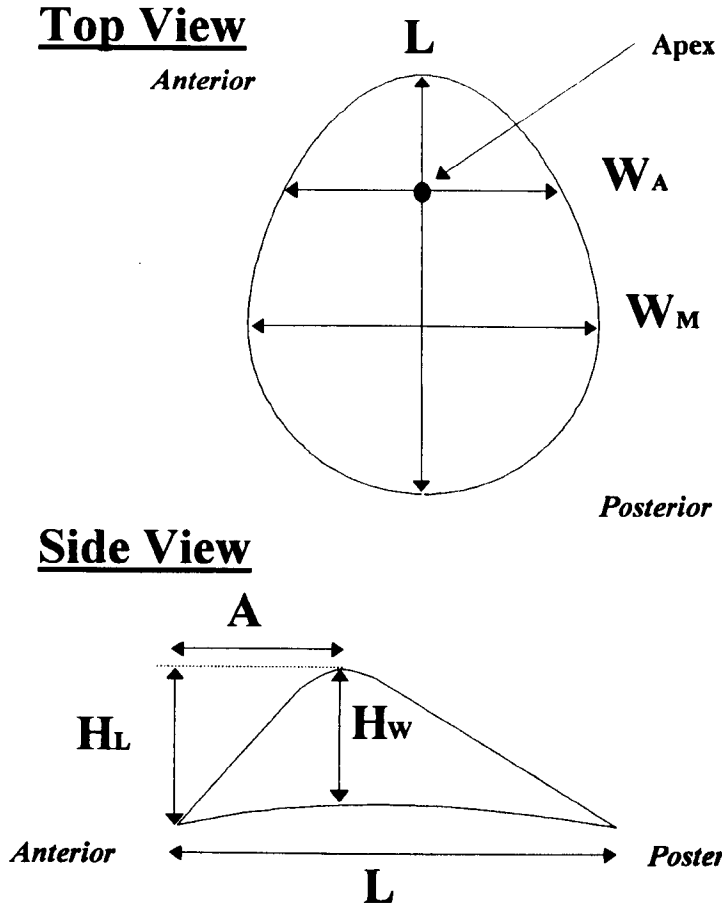
2.2 - Morphological analysis.

A number of soft body characteristics were recorded for each individual: colour of head tentacles, colour of pallial tentacles, presence of orange base to pallial tentacles, presence of stark white pallial tentacles, and colour of the foot muscle.

The radula of each individual was removed by making a cut in the upper lip and drawing it out with fine forceps. The length of the radula was then recorded (to the nearest 0.5mm) using dial calipers. The radula was cleaned in 0.01 M NaOH for one hour, rinsed and stored in 70% ethanol, for possible subsequent morphological examination.

The foot muscle was removed with a scalpel and stored for electrophoretic investigation (See section 2.31 - Electrophoresis, Preparation and storage). The sex, gonad colour and stage of gonad development (in accordance with Orton *et al.*, 1956) were recorded. The shell was cleaned and any epiphytic growth removed from the outside of the shell using a brass wire brush .

For each individual a number of shell parameters were measured, to the nearest 0.5mm, using vernier calipers (Figure 2.3): shell length, maximum shell width, shell width



L	- Shell length.
W_M	- Maximum shell width.
W_A	- Shell width as measured across the shell apex.
A	- Horizontal anterior to apex distance.
H_L	- Shell height measured using the length of the shell as a baseline.
H_W	- Shell height measured using the length of the shell as a baseline.

Figure 2.3 - The measurements taken for each limpet shell. Vernier callipers were used and the measurements recorded to the nearest 0.1mm. Additionally the weight of the shell, in grams, and the length of the radula were recorded.

as measured across the apex, shell height (with the length of the shell as the baseline), shell height (with the width of the shell as the baseline), anterior to apex distance. The two shell width measurements give an impression of how ovate the shell is. Differences between the two shell height measures indicate curvature in the basal plane of the shell. Additionally the shell was weighed to the nearest 0.01g and the radula length measured to the nearest 0.5mm.

In all subsequent data analysis the cube root of the shell weight was used to give a measure linearly-related to the other shell characters. Shells were labelled and stored for future reference.

2.2.1 - Multivariate Techniques

2.2.1.1 - Principal component analysis

The quantitative data was then analysed using principal component analysis (PCA). Details of this method can be found in most multivariate statistics text books (Reyment *et al.* 1984; Manly 1986; Krzanowski 1988). Given a data matrix of r individuals with p variables measured for each, principal component analysis attempts to reduce the essential dimensionality of the data to a fewer number of new variables by orthogonal rotation of the original axes. The result is a new set of variables, or principal components: the first of which attempts to describe as much of the variance in the data as possible; the second describes as much of the remaining variance as possible and so on. This works best for a data matrix with high correlations between variables, resulting in fewer principal components uncorrelated with each other. The first two principal components are linear combinations of the original data that have the largest sample variance associated with them. A plot of these two is then the plane that has the greatest possibility of showing up all the essential features of the original data cloud (Krzanowski 1988).

PCA works best where the variables are comparable in magnitude of variance as well as units of measure (Krzanowski 1988). The original data was therefore transformed using $\log_{10}(x+1)$ and a correlation matrix was submitted to the analysis so that the data set was standardized (Janson and Sundberg 1983; Krzanowski 1988; Sundberg 1988). This transformation also allows size to be separated from shape in the analysis (Reyment *et al.* 1984). The first principal component approximates an isometric size vector if all variable weightings are approximately equal in magnitude and sign, and are equal to $(p)^{-1/2}$ where p is the number of variables (Jolicoeur 1963; Somers 1986). If this holds, then it is argued that the remaining principal components can be regarded as representing variance due to shape differences (Reyment *et al.* 1984).

Discrete partitioning of size and shape by PCA has been disputed by a number of authors (Mosimann 1970; Mosimann and James 1979; Humphries *et al.* 1981). They argued that it is somewhat arbitrary and that the second and subsequent components should be interpreted as a mixture of size and shape. Somers (1986; 1989) proposed a method for extracting an isometric size vector prior to principal component analysis so that the results of the analysis would give 'size-free' shape components. Somer's method has its critics (Rohlf and Bookstein 1987; Sundberg 1989), who suggest that the resultant eigenvectors are not orthogonal and that the technique is perhaps flawed. Due to the uncertainty over Somer's method it was not used. It is generally accepted that the first principal component can be interpreted as size if all variable weightings are approximately equal to $(p)^{-1/2}$ where p is the number of variables (Sundberg 1988) and it is this assumption that shall be used in the analysis.

Principal component analysis does not presuppose any group structure in the data and treats it as one set . This has the advantage of showing any 'natural' group structure and has components that are easier to interpret in terms of size and shape than canonical variate analysis (Humphries *et al.* 1981). It does however mean that the correlation structure of the original data, irrespective of group, determines the orientation of the principal components. Thus PCA does not give maximal separation between groups as is the case with canonical variate analysis (Krzanowski 1988). It is suggested that where a group structure is known then canonical variate analysis is used (Reyment *et al.* 1984). I, however will use principal component analysis to highlight differences in size and shape between groups and demonstrate 'natural' grouping.

Each principal component is a linear combination of the original variables. The eigenvector for each principal component indicates the contribution of each shell parameter to the variance explained by that component. The elements of an eigenvector with greatest absolute magnitude indicate which variables are contributing most to the variance described by that principal component. The sign of an element in the eigenvector indicates a positive or negative correlation between that variable and the principal component. Significant differences between groups in the data were tested for with analysis of variance (ANOVA) and student-Newman-Keul (SNK) tests.

2.2.1.2 - Canonical discriminant analysis

Canonical discriminant analysis is a well established technique used on multivariate normal data where information on subgroups is available. For full details of this technique see Campbell & Atchley (1981); Reyment *et. al.* (1984); Krzanowski (1988). The major aim of the technique is to provide a low dimensional representation of the data that highlights, as accurately as possible, the true differences existing between the groups (Krzanowski 1988). It uses the group information to maximise the between- and within-group variances, thus attempting to give a maximal separation of the groups. This approach can be considered as a two stage rotation. The first, a principal component analysis on the original variables. The second involves an eigenanalysis of the variation between the group means, for the variables from the first stage of the analysis (Campbell and Atchley 1981). Characters with high positive within-group correlation and negative between-group separation, and *vice versa* will provide better separation (Lubischew 1962).

Although superficially similar to principal component analysis, the two techniques are profoundly different in many ways. One important difference is that, unlike principal component analysis, the axes in canonical discriminate analysis are non-orthogonal. Thus the canonical space is a distortion of euclidian space. Distances in this space are therefore not euclidian, whereas principal component analysis is merely a rotation of the original axes preserving orthogonality between axes and not altering the euclidian distance between points.

There are a number of ways of interpreting the results of canonical discriminate analysis. The most usual, and the one that shall be used here, is the absolute magnitude of the standardized canonical variate coefficients (Campbell and Atchley 1981; Reyment *et al.* 1984). The coefficients are standardized to put all variates on a comparable footing as regards within-group variability (Krzanowski 1988). Those coefficients for a canonical variate that are of a large magnitude relative to the rest, are the ones important in separating the groups along that axis.

The maximum dimensionality for a canonical variate representation is s where:

$s = \min(p, g-1)$

p = number of variables

g = number of groups.

Thus where only two groups are defined (e.g. mesolittoral and infralittoral limpets) the maximum number of canonical variates is one and so the results are presented as frequency distributions along canonical variate 1. Where the data are separated into the three or more groups the maximum possible number of canonical variate is greater than one. In this case bivariate plots were used to visualise the relationship between groups in multivariate space.

On the canonical variate graphs group means and 95 % confidence regions are included. The 95 % confidence region is the tolerance region within which 95 % of the whole population, of which the group is a sample, is expected to lie. As the original data is normally distributed, the canonical variates will also be jointly normal. Within each group canonical variates are uncorrelated and have unit variance. Coupled with normality, this implies within-group independence and unit variance for the canonical variates (Krzanowski 1988). The tolerance regions can therefore be generated from a χ^2 distribution independent of the sample size. For a bivariate plot the $100(1-\alpha)$ confidence region has a radius r where

$$r = (2,\alpha,2)^{1/2}$$

So for a 95 % confidence region this will be $(4.61)^{1/2} = 2.15$.

Further, the confidence region for the population mean will be $2.15 \div \sqrt{n}$ where n is the number of individuals in that group.

Where a single axis plot is used, as for the data set differentiated by shore zone, the confidence region $100(1-\alpha)$ is r where

$$r = (2,\alpha,2)^{1/2}$$

So for a 95 % confidence region this will be $(2.106)^{1/2} = 1.645$.

Again the tolerance for the population mean is $1.645 \div \sqrt{n}$ for each group, where n is the number of individuals in that group.

Dendrograms have been used in Chapters 4,5 and 6 to visually illustrate the distances between groups in canonical space. The dendrograms are based on the squared distances in canonical space and are constructed using the unweighted pair group method with recomputation of coefficients by arithmetic averaging (UPGMA, see Sneath and Sokal 1973)

2.2.1.3 - Canonical Discriminate function analysis

Canonical discriminate analysis is used to maximise the differences between groups and give account of the characters primarily responsible for these differences. Null hypothesis testing for equality between groups and group means can be applied but these hypotheses are limited. The fact that the group structure of the data set is given suggests there are some differences between the groups, the canonical discriminate analysis being a test of the ability to detect these differences with the variables measured.

A discriminate function for the data can be used to provide more information on the differences between the groups. A discriminate function analysis provides an allocation rule based on the original data set that assigns individuals to one of the groups. A way of analysing group differences is then to look at the proportion of correctly assigned individuals for each group, and into which group incorrectly allocated individuals are placed. For full details of this technique you are referred to Cooley & Lohnes(1971) and Krzanowski(1988). The prior probability of membership to any group was assumed to be equal, there being no evidence to suggest that it should be otherwise.

Linear or quadratic functions may be used in discriminate function analysis (Krzanowski 1988). A quadratic function has the advantage that it allows for different dispersions in the populations (Krzanowski 1988). Relative to a linear function, a quadratic function is unstable and the population function derived can fluctuate wildly dependent upon the population sample data set (Lachenbruch *et al.* 1973; Krzanowski 1988). It was decided to use linear discriminate functions due to their markedly increased stability over quadratic functions.

The simplest method of estimating the performance of a discriminate function is finding the apparent error rate using the resubstitution method. This will use the original data as a training set and then resubstitute this data into the resultant discriminate function and find

its ability to correctly assign them. This is a biased method of estimating error rates, as the same individuals used to establish the allocation rules are then used to establish its performance. This means that as the function gives maximal separation between the training sets, then within the population these training sets are those with the lowest chance of misallocation. The resubstitution method will therefore underestimate the error rate for allocation of other individuals from the populations (Krzanowski 1988).

To avoid the bias caused by using the same data to make and then test the discriminate function, a sample-splitting method can be used whereby the groups are randomly split into two portions. One portion is then used to create the allocation rule and the other portion used to establish its ability to assign individuals to the correct groups. This has the advantage that separate data is being used for the two parts of the method. It does present other problems, the original data sets must be large so that there are enough individuals within a sub-group to avoid large sampling fluctuations in either the allocation rule or the performance analysis.

The method of discriminate function analysis that will be used is a combination of the resubstitution and sample-splitting methods called cross-validation (Lachenbruch and Mickey 1968). The allocation rule is determined using the whole data set minus one individual, the resultant rule is used to classify that individual. This process is then repeated omitting each of the individuals in the data set in turn. The resulting apparent error rate estimates will be larger than obtained from a resubstitution procedure and the variance will also be greater. The error estimates will, however, be more realistic indicators of the ability of the function to correctly assign individuals from outside the training sets to the correct population.

2.2.1.4 - Morphometric data analysis

The three multivariate techniques used in this study were carried out using SAS/STAT release 6.03 (SAS Institute Inc, 1988): principal component analysis using SAS PROC PRIN; canonical variate analysis using SAS PROC CANDISC and discriminante function analysis using SAS PROC DISCRIM.

2.2.2 - S.E.M. analysis of the radula structure

Analysis of the radula structure was carried out on selected stored specimens of the species found at each site. The radula was removed from 70% ethanol and air dried. It was cut into short sections and mounted on a stub using double-sided cellotape. Care was taken to ensure the radula was securely positioned, with the basal strip of the radula attached to the cellotape, but radula manipulation was minimised to reduce excess damage to the teeth. The radula was examined using a Phillips S.E.M.

2.3 - Electrophoresis

2.3.1 - Preparation and storage.

For the electrophoretic work the foot muscle tissue of the limpet was used. The muscle was removed from live limpets; only healthy individuals were sampled. The muscle was cut away from the other tissue using a scalpel and any mucus was wiped off. The muscle was then sealed in a plastic bag and placed in liquid nitrogen (-196°C boiling point), to freeze the tissue rapidly and so reduce protein lysis. Samples were then transferred to a freezer and stored at -20°C until used for electrophoresis.

2.3.2 - Electrophoresis.

The genetics of the samples of limpets was examined using standard horizontal starch gel electrophoresis (following Selander *et al.* 1971; Harris and Hopkinson 1977; Ferguson 1980). A number of different buffer systems over a range of pH were tried. Although a number of combinations gave adequate results, Tris citrate pH 8.0 gave sufficient satisfactory results for it to be adopted as the sole buffer system.

A small portion of the foot muscle (approx 30 mm^3) was placed in an ependorf tube along with 1mg of acid washed sand to aid the breakup of the tissue and 50ul of a mercaptoethanol grinding buffer (Baverstock & Adams, 1984). The tissue was well broken up and the samples were spun out in a Hawksley micro-haemocrit centrifuge for 5 minutes. This separated out mucus and tissue fragments, leaving a clear protein-rich supernatant. The use of the centrifuge was found to reduce streaking in the gel caused by blockage of the gel ma-

trix by mucus, which interfers with protein migration. The supernatant was then absorbed onto a 4 x 12mm filter paper wick (Whatman 3mm).

All gels were made up using 47.5g of starch (Sigma Chemicals, Poole, Dorset) and 380 ml of Tris citrate buffer pH 8.0. This mixture was heated in a conical flask over a Bunsen burner until the starch was just at boiling point, but was not allowed to boil (approx 10 mins). The flask was constantly agitated to ensure homogeneity of the mixture and to prevent scorching of the starch.

The mixture was degassed using a water powered vacuum pump for 30 seconds. The mixture was then poured onto a glass plate (210x180mm) bounded by a perspex frame 10mm deep (180x150mm internal dimensions). Any remaining bubbles were removed with a spatula and a second glass plate placed on top of the frame; care was taken not to trap air.

The gel was allowed to cool overnight then the top plate and frame were removed and the gel cut along its length 2cm from one edge, ready for the loading of samples. The sample wicks were loaded, 18 to a gel, in blocks of six, separated by a wicks soaked in a ferritin solution (concentration) to act as markers. The frame was then replaced and the gel moved to a prepared buffer tank.

Shannon model 600 buffer tanks were used and Shannon 'Vokum 400' power packs. The buffer tank was cleaned, dried and filled with 600ml of Tris citrate pH 8.0 electrode buffer. The gel was put in the tank with the wicks at the anodal side, as it was found that most of the enzymes under study ran cathodally. The cloths used to form the salt bridge were placed along the length of the gel 10 cm apart, with the anodal cloth being placed just behind the wicks. The tank was then run at 160V for 6 hours, with the power pack set to self-regulate the voltage. During this time a covered plastic box, containing crushed ice floated in water, was placed on the gel, between the two wicks, separated from the gel by a layer of clingfilm. The whole apparatus was kept in the fridge and the ice and water in the plastic box were changed after three hours. These precautions were taken to reduce the heating effect of the current being passed through the gel. Heat causes protein breakdown and distortion of the gel. 15 minutes into the run the wicks were removed from the gel. This was found to reduce streaking when the gels were subsequently stained.

After six hours the gel was removed from the tank. The distance travelled by the four feratin markers was recorded. The gel was then sliced using a Shannon gel slicer into 10 slices. The top slice was discarded as gel surface effects could distort results taken from this slice.

2.3.3 - Staining

The gel slices were stained using standard solutions (Shaw and Prasad, 1970; Schaal and Anderson, 1974; Harris and Hopkinson, 1978). Staining took place in plastic trays which were covered and incubated at room temperature until the stain developed. This took between 5 minutes (PGI) and 24 hours (ME). Staining patterns were recorded as they developed.

2.3.4 - Electrophoretic data analysis

The analysis of electrophoretic results was based on the genotype frequencies of the loci for each species at each site. Genetic statistics were calculated using BIOSYS-1 Release 1.7 (Swofford and Selender, 1989).

3 - Genetics and morphometric variation between mesolittoral and infralittoral *Patella caerulea* (Linnaeus, 1758) from Crete and Cyprus

3.1 - Introduction

Patella caerulea shows an essentially bimodal distribution with tidal height on rocky shores of the Mediterranean (Davies 1969a). There is a group concentrated in the lower mesolittoral zone (Della Santina *et al.* 1992), which is bounded above by *P. rustica* in the supralittoral and below by *P. aspera* in the infralittoral fringe (Della Santina *et al.* 1992). Mesolittoral groups are found on most rocky shores of the Mediterranean. In some areas there is a second, physically separate, group in the infralittoral zone. This second group most often occurs in infralittoral regions where there is a break in the heavy algal cover (C.Nobles, *pers. obs.*). This group is bounded above by the *P. aspera* in the infralittoral fringe and can extend down to the lower limit of the infralittoral zone (Sella and Bacci 1971). Davies (1969a) described the presence of subtidal *P. caerulea* down to 4m below mean sea-level. This group comprised individuals larger than those found in the mesolittoral zone and had a depressed shell having large ridges and a more crenulated margin. He further showed that the infralittoral form had a smaller R/C ratio than did the mesolittoral form (See Chapter 1 Section 1.2). The greater shell height in mesolittoral individuals was attributed to an adaptational change that reduces water loss when exposed to the air (Davies 1969b). The reduction in shell height relative to length at lower shore levels for species of limpet has been attributed to various factors: water turbulence and wave action; (Russell 1907; Ebling *et al.* 1962; Walker 1972; Berry & Rudge 1973) and desiccation (Orton *et al.* 1956; Davies 1969a, 1969b; Sella and Bacci 1971).

Bannister (1975) also examined the biometrics of *P. caerulea* but confined his study to the mesolittoral zone. The problem with the work of Bannister and of Davies is that neither author acknowledges the presence of *P. aspera* in the study. There is therefore the danger that what is described as *P. caerulea* is in fact a combination of *P. caerulea* and *P. aspera*. Davies acknowledges finding anomalous individuals - 'Occasionally specimens were found with a orange foot, very closely resembling *P. aspera*'. Furthermore it is known that *P. as-*

pera is present at Manoel Island in Malta where Bannister collected *P. caerulea* from (C.Nobles, *pers. obs.*).

Sella & Bacci (1971) looked at relationships between mesolittoral and infralittoral populations of *Patella* in the Mediterranean. Limpets were collected from two sites in Italy. Mesolittoral samples were collected 50cm above and below the low water level and were found at a density of not less than 40 - 50 individuals per m^2. The infralittoral zone was deemed to be at a depth of 2m and below, samples were collected from between 3 and 5m and between 6 and 9m depth. At one site collections were made down to a depth of 26m. Infralittoral *P. caerulea* were found at a density of no greater than three individuals per m^2. This work scored individuals on the basis of sets of contrasting characters (Bacci and Sella 1970; Sella and Bacci 1971) and measured the maximum basal diameter **D** (called shell length in my study) and shell height **h** of individuals. The work showed that infralittoral *P. caerulea* tended to be polygonal in shape (*Subplana* morphotype) with marked shell ribs. The **D/h** ratio was significantly higher in infralittoral populations showing that infralittoral individuals tend to be lower for a given shell length than mesolittoral individuals. They suggested that the depressed shell is a genetically determined trait which allows individuals to survive in the infralittoral zone, although no direct evidence is presented for this. Sella & Bacci (1971) suggested that the infralittoral population is maintained by migration of larger individuals from the mesolittoral, where the depressed subplanar shape is present with a low frequency (10 - 30 %). The low frequency in the mesolittoral zone also explaining the lower population density in the infralittoral. Christiaens (1973) expresses doubt about the correct identification of some of the *P. aspera* and *P. caerulea* in the work of Bacci and Sella (1970), which may affect the validity of the biometric work they carried out.

Further work on the biometric relationships between mesolittoral and infralittoral populations of *P. caerulea* (Sella 1976) examined radula length, shell length and shell height. Model I linear regression was used to show differences in radula length/shell length (R/C fraction) and radula length/shell height relationships between *P. caerulea* from the two zones. Both ratios showed a decrease from the mesolittoral to infralittoral zone. The relatively shorter radula is accompanied by a larger shell base area in infralittoral limpets. Sella (1976) attributed this to reduced desiccation stress (Moore 1934; Davies 1969b; Sella and Bacci 1971; Sella 1976). She also suggested that the reduction of radula length with depth

may be a genetic rather than phenotypic adaptation, again though there is no direct evidence for this.

Work on the shell morphology of *Nacella concinna* (Strebel 1908), an antarctic limpet sampled from Signy Island (Nolan 1991) shows a reduction in shell height in sublittoral individuals (for a length of 25mm an average animal will be 8.07mm high in the littoral and 6.62mm high in the sublittoral). Although markedly different to the Mediterranean environment in many respects it is clear that the littoral zone in the Antacrtic is highly stressful and similar adaptational forces are acting (or at the least different forces are acting in the same direction) to produce individuals with a relatively taller shell higher on the shore. Further work on this species including shell width data (Beaumont and Wei 1991), showed similar results with sublittoral individuals having a larger basal area and more depressed profile than littoral individuals. Unfortunately no data is presented for radula length so the comparison cannot be extended to include this parameter.

Cellana radiata (Born), a tropical species of limpet found in India shows similar differences in shell shape to other species (Moore 1934; Ebling *et al.* 1962; Davies 1969; Branch and Marsh 1978)with respect to height on the shore. Work on high and low shore forms showed low shore individuals to be larger, with a low spire and the apex positioned more towards the anterior of the shell (Rao and Ganapati 1971).

Most of the work on shell shape has been based on averaged ratios and model I linear regression. Where looking at shape difference, both these methods (which are in essence the same) have severe shortcomings (See chapter 1 section 1.2).

In investigating the biochemical genetics of *P. caerulea* work has concentrated on interspecific variation and taxonomy (Hatch 1977; Wilkins 1977; Badino and Sella 1980a, 1980b; Gaffney 1980; Sella and Badino 1980, 1982; Lavie *et al.* 1987; Sella *et al.* 1989) and intraspecific variation between geographically separate populations (Sella *et al.* 1985; Badino *et al.* 1986).

The genetic relationship between mesolittoral and infralittoral *P. caerulea* has not been looked at. In *P. aspera* the intertidal and subtidal morphs in the Azores were shown to be conspecific (Côrte-Real 1992), the observed differences in shape were attributed to morpho-

logical plasticity. In *Nacella concinna* there was no measureable genetic differentiation between intertidal and subtidal populations (Beaumont and Wei 1991) even though they were morphologically distinct (Nolan 1991). It was suggested that the observed morphological differentiation was evidence of phenotypic plasticity.

The aim of this work is to reinvestigate, employing current multivariate techniques (Janson and Sundberg 1983a, 1983b; Sundberg 1988; Furman 1990; Mc Donald *et al.* 1991) the differences in shape between mesolittoral and infralittoral populations of *P. caerulea*. These techniques can be used to establish the degree of difference in shell shape and the form of that difference between *P. caerulea* from the mesolittoral and infralittoral zones. Biochemical genetics will then be employed to establish the degree of genetic differentiation between mesolittoral and infralittoral populations. The combination of the morphological and genetic analysis will hopefully shed light on the origins of the the two separated groups.

3.2 - Materials and methods

3.2.1 - Collection of samples

Samples of limpets were collected from Irepetra in Crete and Limassol in Cyprus (See Figure 2.2). To minimise environmental differences,the two sites chosen were both moderately exposed breakwaters (See Chapter 2 Section 2.1). At each site a sample of approximately 100 individuals was taken from each of the two shore levels. Mesolittoral samples were collected from above the red algal turf zone that marks the top of the infralittoral fringe. Infralittoral samples were collected by snorkelling and came from between 1m and 6m below the infralittoral fringe.

3.2.2 - Qualitative soft body characters

A number of soft body characteristics were recorded for each individual: colour of head tentacles, colour of pallial tentacles, presence of orange base to pallial tentacles, and colour of the foot muscle. The sex of each individual was also recorded, along with the gonad stage (Orton *et al.* 1956) and colour.

3.2.3 - Morphological analysis

For each individual a number of shell parameters were measured, to the nearest 0.5mm, using vernier calipers (Figure 2.3): shell length, maximum shell width, shell width as measured across the apex, shell height (with the length of the shell as the baseline), shell height (with the width of the shell as the baseline), anterior to apex distance. The two shell width measurements give an impression of how ovate the shell is. Differences between the two shell height measures indicate curvature in the basal plane of the shell. Additionally the shell was weighed to the nearest 0.01g and the radula length measured to the nearest 0.5mm. Shells were labelled and stored for future reference.

In analysing the data, three different group structures were used:

1 - Four separate groups: Irepetra mesolittoral, Irepetra infralittoral, Limassol mesolittoral and Limassol infralittoral.

The data could be regarded as a crossed two-factor design,with the factors being zone on the shore and geographic location. Thus to aid visualisation of the results, the other two group structures were:

2 - Two groups, with the samples from the same zone pooled (Mesolittoral and Infralittoral).

3 - Two groups, with the zones within a site pooled: (Irepetra and Limassol).

These three group structures were used in the presentation of the principal component analysis results. The same three group structures were also used as the groupings for canonical discriminant analysis. Before analysis the data were $\log_{10}(x+1)$ transformed to standardize the variance. Two-way ANOVA was used to analyse the principal components, using shore zone and geographic location as the two factors.

The whole analysis was also repeated on a data set with the radula length not included in the analysis, there are a number of reasons for this:

1 -. The radula length is the variable that has the lowest correlation coefficients with other variables. As such it will be the variable that contributes most to the total vari-

ance of the data set. This variance is not necessarily inter-group variance and could be intra-group variance that obscures patterns in the data pertaining to shell shape rather than highlighting them.

2 - Though radula length has been shown to vary with zone on the shore (Moore 1934; Ebling *et al.* 1962; Davies 1969a) and between species (Fischer-Piette 1935; Fischer-Piette and Gaillard 1959; Christiaens 1973) it is not a shell shape character. It was decided to exclude radula length and see whether the shell shape alone was sufficient to discriminant between groups.

3 - Excluding radula length allows the possibility of comparing the results with data from other sites where the limpets are collected non-destructively. Also museum specimens could be measured and compared with this study.

3.2.4 - Electrophoresis

Electrophoresis on the samples was carried out following the methods described in detail in the general materials and methods (Chapter 2, section 2.3). In brief, for the electrophoretic work the foot muscle tissue of the limpet was used. The muscle was removed from live limpets, only healthy individuals were sampled. The genetics of the samples of limpets was examined using standard horizontal starch gel electrophoresis (following Selander *et al.* 1971; Harris and Hopkinson 1977; Ferguson 1980). Tris citrate pH 8.0 was used as the gel buffer. The gel slices were stained using standard solutions (Shaw and Prasad, 1970; Schaal and Anderson, 1974; Harris and Hopkinson, 1978). Thirteen loci were assayed : *Pgi-1*, *Pgm-1*, *G3pdh*, *Pgdh*, *Np*, *Mpi-1*, *Mdh-1*, *Mdh-2*, *Ald-1*, *Idh-1*, *Idh-2*, *Me-2* and *Got*.

The analysis of electrophoretic results was based on the genotype frequencies of the loci for each species at each site. Genetic statistics were calculated using the program BIOSYS-1 Release 1.7 (Swofford and Selender, 1989).

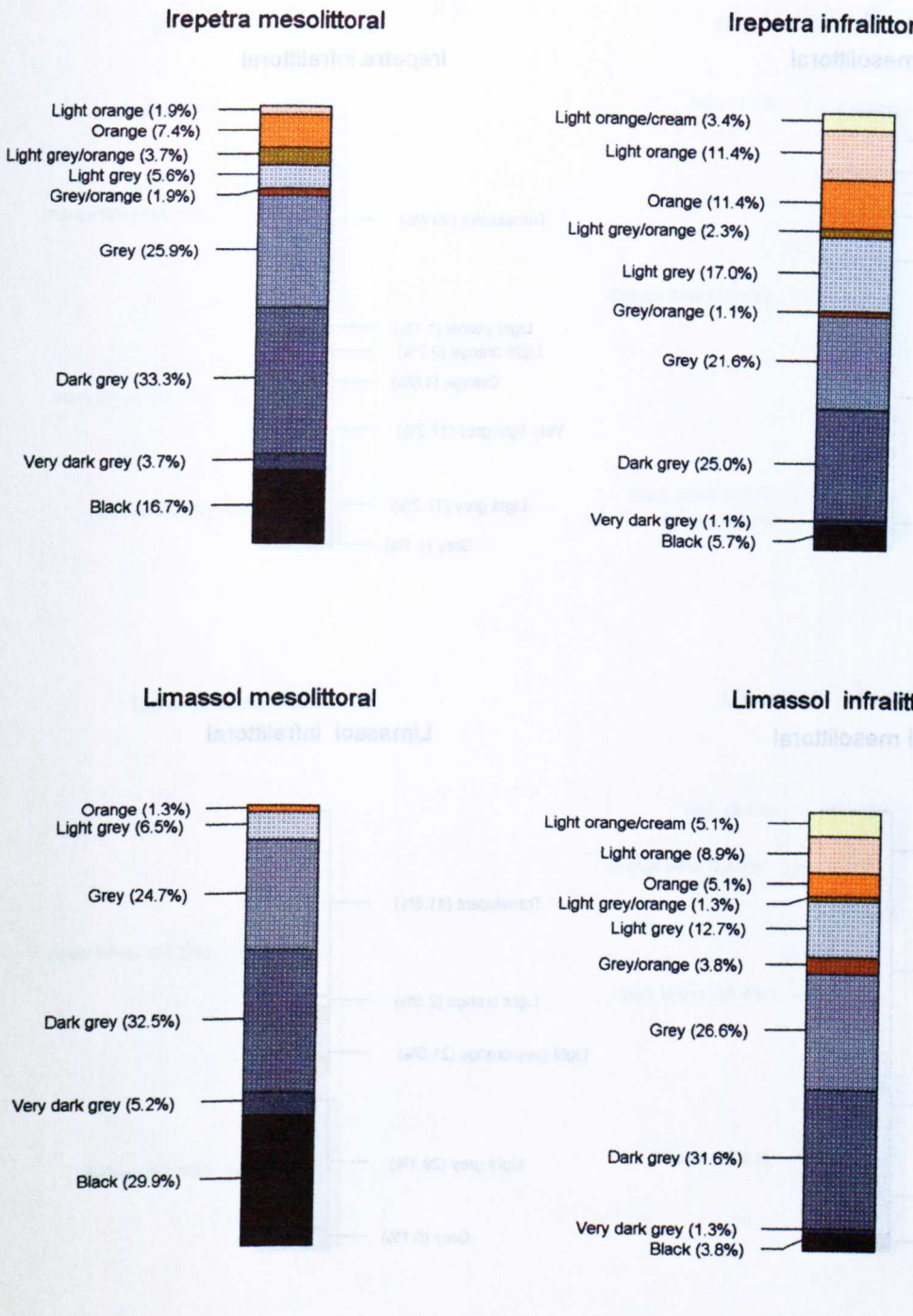


Figure 3.1 - The proportion of individuals of different foot colour for mesolittoral and infralittoral *P.caerulea* from Irepetra and Limassol. Proportions given as percentage of the group (sample sizes are: Irepetra mesolittoral=55, Irepetra infralittoral=89, Limassol mesolittoral=80 and Limassol infralittoral=79).

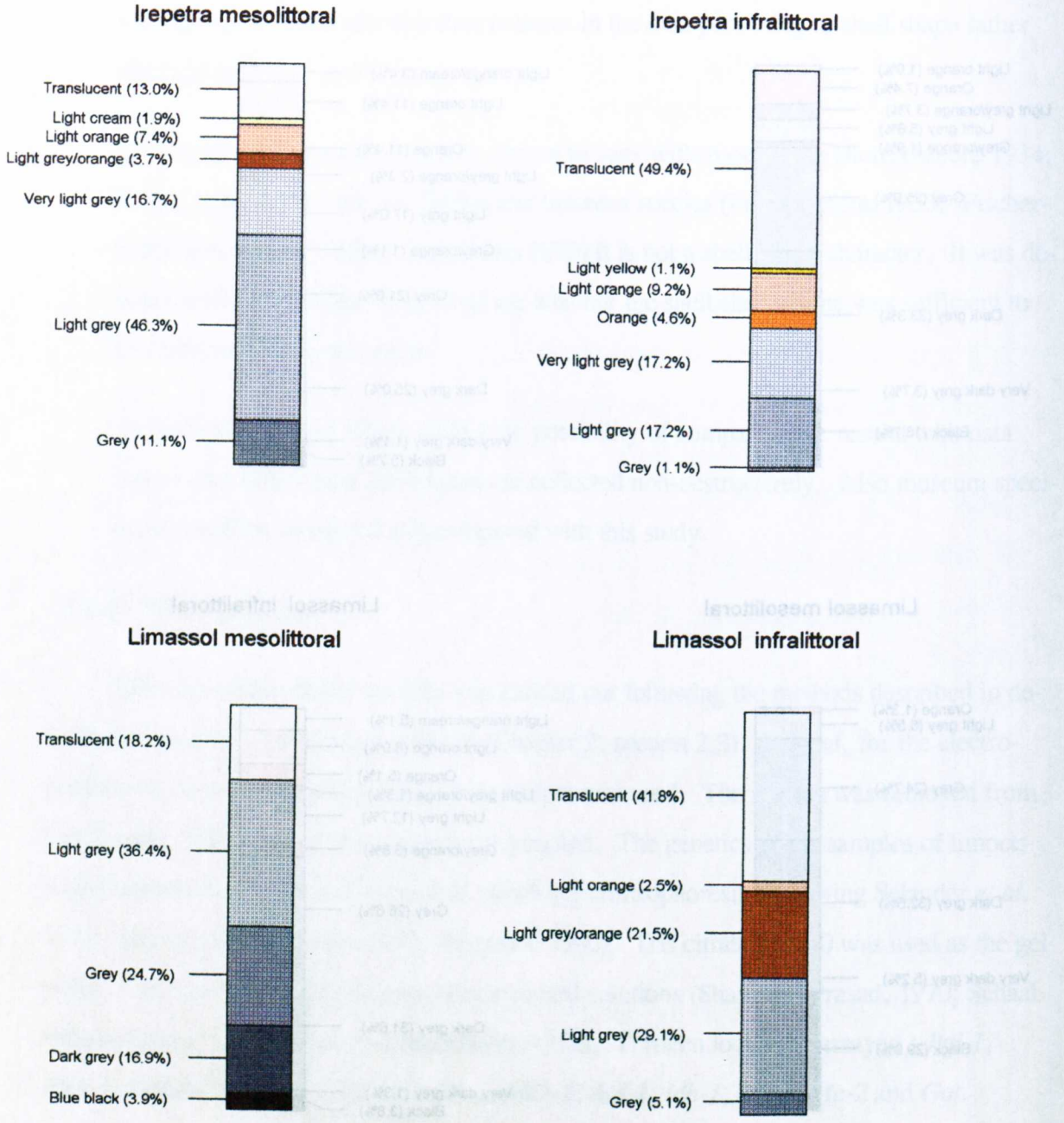


Figure 3.2 - The proportion of individuals of different head tentacle colour for mesolittoral and infralittoral *P.caerulea* from Irepetra and Limassol. Proportions given as percentage of the group (sample sizes are: Irepetra mesolittoral=55, Irepetra infralittoral=89, Limassol mesolittoral=80 and Limassol infralittoral=79).

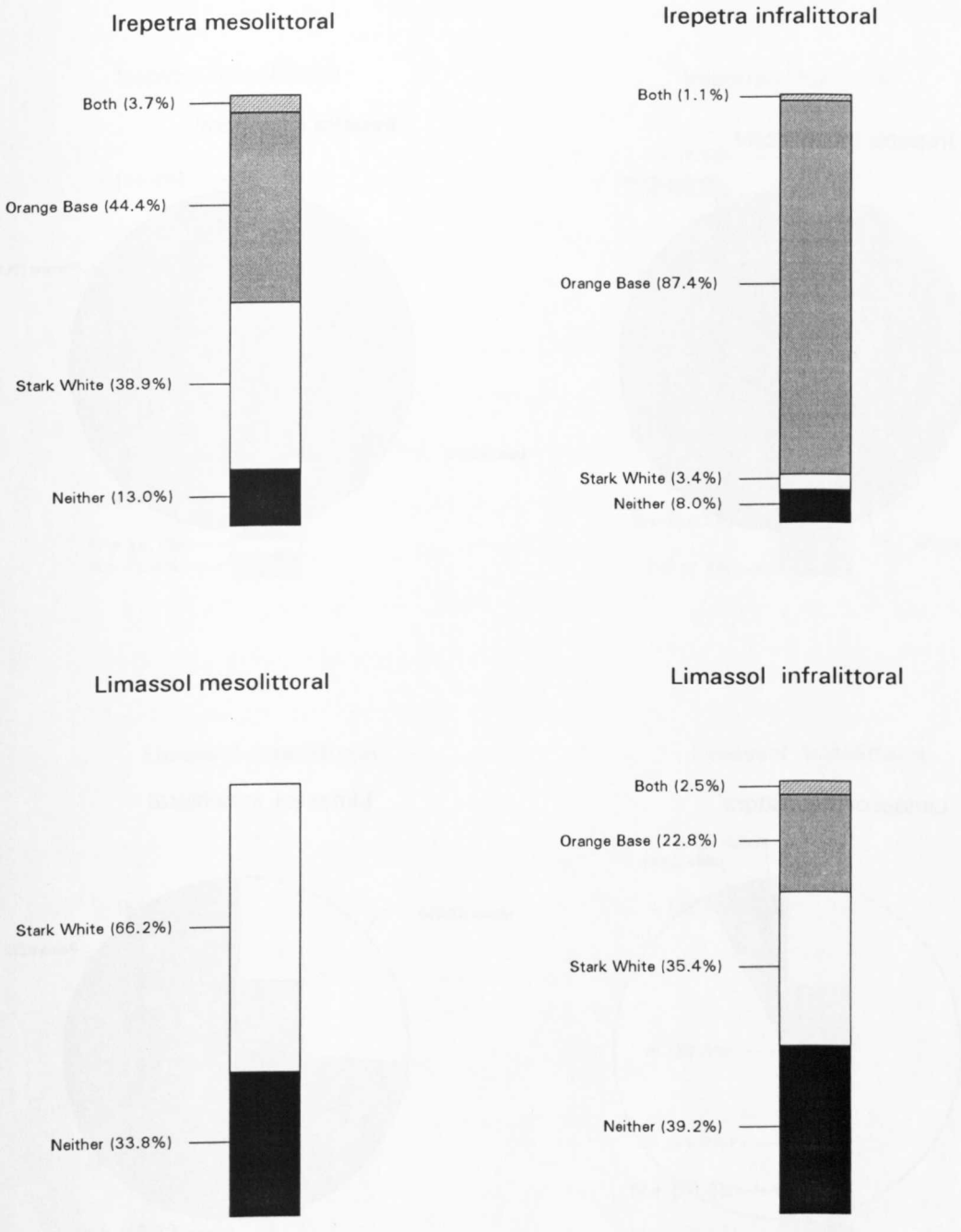


Figure 3.3 - The proportion of individuals displaying two colour characteristics of the pallial tentacles for mesolittoral and infralittoral *P.caerulea* from Irepetra and Limassol. Proportions given as percentage of the group. Orange Base indicates an orange to brown colouration, to the base of the pallial tentacles. Stark white indicates that the tentacles are a clear and bright white colour. Both indicates individual with both characters. neither indicates individuals with neither characteristic (sample sizes are: Irepetra mesolittoral=55, Irepetra infralittoral=89, Limassol mesolittoral=80 and Limassol infralittoral=79).

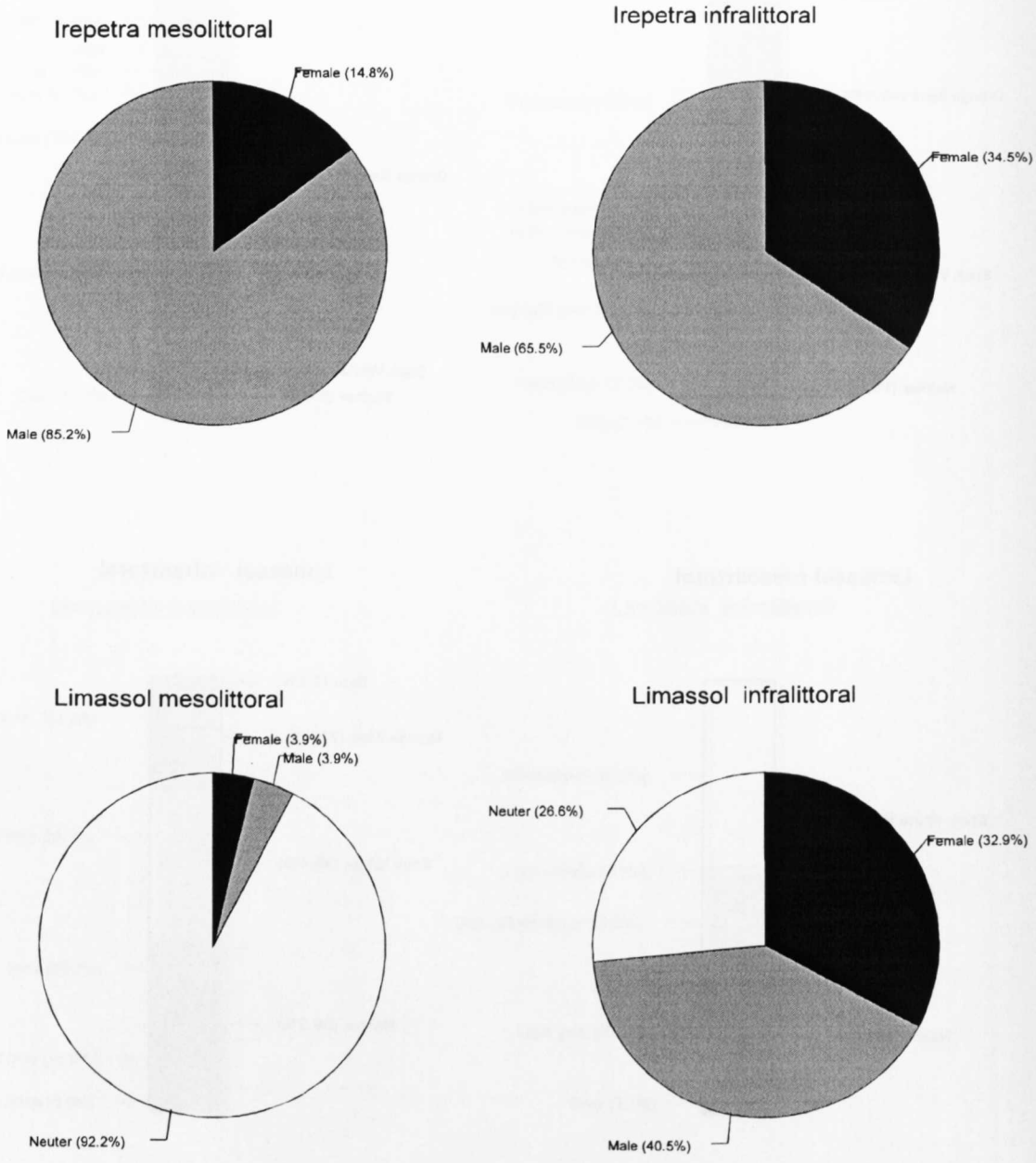


Figure 3.4 - The proportion of males females and neuters for mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol. Proportions given as percentage of the group (sample sizes are: Irepetra mesolittoral=55, Irepetra infralittoral=89, Limassol mesolittoral=80 and Limassol infralittoral=79).

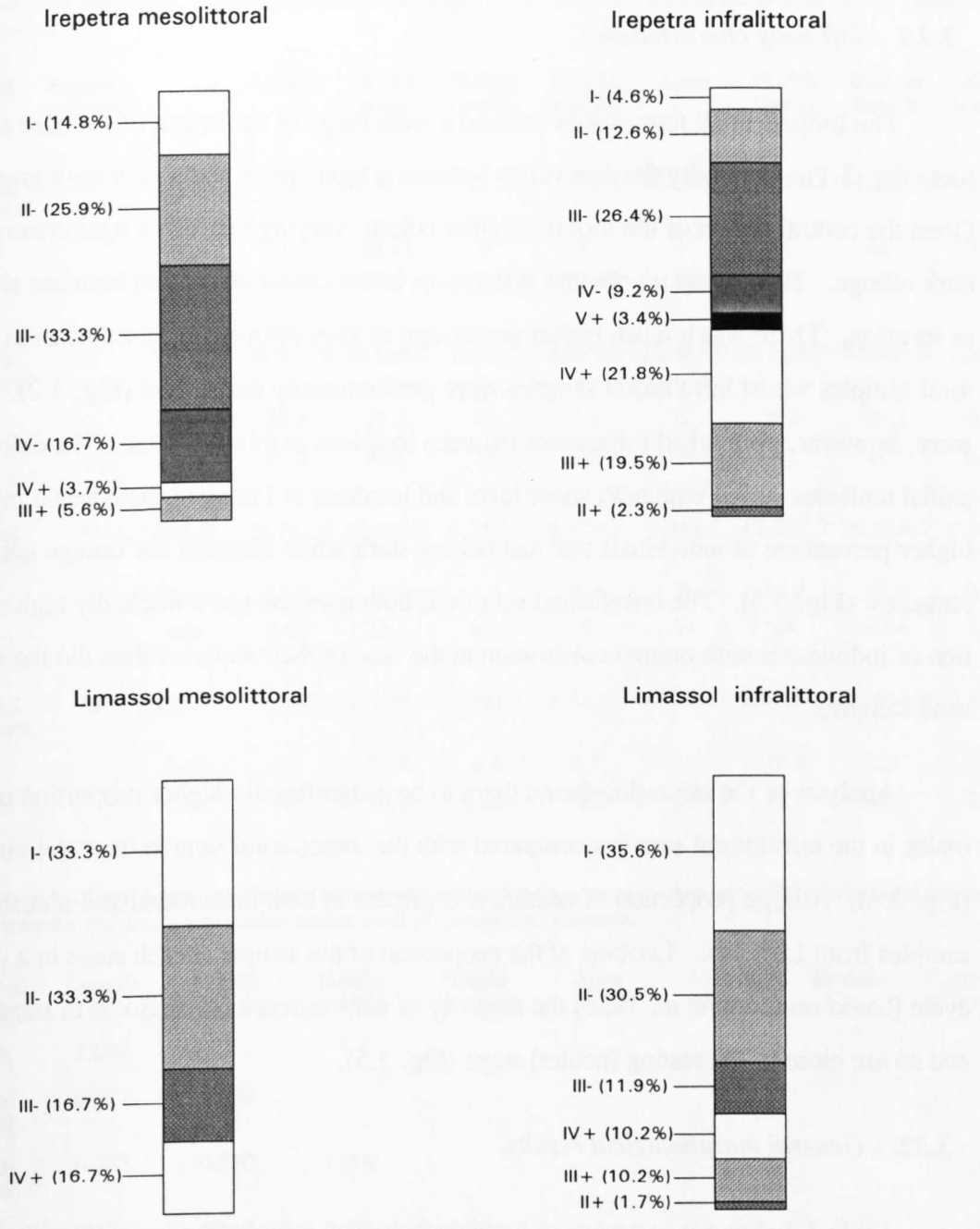


Figure 3.5 - The proportion of individuals of different stages of the gonad cycle (Orton et al., 1956) for mesolittoral and infralittoral *P.caerulea* from Irepetra and Limassol. Proportions given as percentage of the group (sample sizes are: Irepetra mesolittoral=55, Irepetra infralittoral=89, Limassol mesolittoral=6 and Limassol infralittoral=58).

3.3 - Results

3.3.1 - Soft body characteristics

The limpets in all four groups showed a wide range of coloration of the base of the foot (Fig. 3.1). Generally the foot varies between a light cream and a very dark grey/black. Often the central region of the foot is a lighter colour, varying between a light cream and a dark orange. There were no obvious differences in the colour of the foot between shore level or location. There was a much higher percentage of grey coloured head tentacles in mesolittoral samples whilst infralittoral samples were predominantly translucent (Fig. 3.2). There were, however, no marked differences between locations in this character. The colour of the pallial tentacles varied with both shore level and location: at Limassol there was a much higher percentage of individuals that had neither stark white tentacles nor orange bases to the tentacles. (Fig. 3.3). The infralittoral sample at both sites showed a markedly higher proportion of individuals with orange colouration at the base of their tentacles than did the mesolittoral samples.

Analysis of the sex ratios shows there to be a significantly higher proportion of females in the infralittoral samples compared with the mesolittoral sample from the same site (Fig. 3.4). A large proportion of neuters was present in both infralittoral and mesolittoral samples from Limassol. Looking at the proportion of the sample at each stage in a gonadic cycle (based on Orton *et al.* 1956) the majority of non-neuters at Limassol is in stage I^- or II^- and so are close to the resting (neuter) stage (Fig. 3.5).

3.32. - General morphological results.

Table 3.1 shows a summary of the morphological information for each of the four groups. The sample size ranges from 55 to 89 individuals. As a general observation the average value for all the shell measures is larger in the infralittoral groups than in the mesolittoral groups. Secondly the average value for shell measures from either Irepetra group is higher than that of the corresponding Limassol group.

Table 3.1 - Simple summary statistics for the eight variables from each of the four groups: Irepetra mesolittoral, Iretpetra infralittoral, Limassol mesolittoral and Limassol infralittoral, showing the number collected at each site, mean, standard deviation and range of each variable. Except for weight (g) all measurements are in mm.

Site and zone	Number collected		Length	Width (max)	Height (width)	Height (length)	Apex	Width (apex)	Radula length	Shell weight
Ireperta mesolittoral	55	$\bar{x}$±S.D.	26.7±4.5	23.4±3.9	7.1±1.5	7.4±1.6	10.7±2.2	22.7±4.1	34.8±5.8	1.1±0.6
		max.	21.5	18.21	4	4	8	16.7	22.7	0.38
		min.	45.6	38.7	11.6	12.4	21	38.6	49	4.42
Irepetra infralittoral	89	$\bar{x}$±S.D.	34.9±5.8	30.5±5.4	7.9±1.8	8.8±2.0	14.4±3.0	29.0±5.7	37.8±5.9	2.2±1.1
		max.	22.8	18.8	4.1	4.4	6	9.2	23.2	0.57
		min.	47.3	41.6	14	15.9	21	40	51.3	6.04
Limassol mesolittoral	80	$\bar{x}$±S.D.	22.7±2.6	19.8±2.2	6.2±0.9	6.4±0.9	8.9±1.1	18.8±2.2	30.9±4.5	0.6±0.2
		max.	15.9	13.6	4.4	4.4	6	13.3	18.8	0.21
		min.	31.9	25.6	8.1	9	11	24	40.4	1.43
Limassol infralittora	79	$\bar{x}$±S.D.	32.5±3.5	27.6±3.1	7.7±1.1	8.3±1.2	12.8±1.7	26.1±3.2	35.8±4.9	1.6±0.6
		max.	25	21.8	4.8	5.3	9	18.8	22	0.65
		min.	42	36.8	10.4	11.2	17	34.8	46.2	3.78

Table 3.2 - Correlation coefficients between variables for the complete data set. Data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize variance.

Measured character	Length	Width (max)	Height (width)	Height (length)	Apex	Width (apex)	Radula	Cube root of weight
Length	1.000							
Width (widest)	0.977	1.000						
Height (width)	0.762	0.770	1.000					
Height (length)	0.816	0.814	0.971	1.000				
Apex	0.945	0.945	0.767	0.821	1.000			
Width (apex)	0.947	0.973	0.791	0.825	0.933	1.000		
Radula	0.692	0.696	0.790	0.804	0.672	0.702	1.000	
Cube root of weight	0.961	0.965	0.844	0.884	0.939	0.948	0.737	1.000

The correlation coefficients between the eight variables for the whole data set show high correlations between the transformed shell measures (Table 3.2). The important point to note from this table is that the lowest correlation coefficients are between radula length and all the other elements. This lower correlation means that radula length will contribute a greater proportion of the total variance in the data set than will any of the other elements. This is one of the reasons for doing one set of the multivariate analysis with radula length excluded.

3.3.3 - Principal component analysis

The first four principal components explain 98.5% of the variance in the data (Table 3.3), of which 86.8% is explained by the first principal component. Table 3.4 shows the eigenvectors for the first four principal components. All the elements of the eigenvector for principal component 1 are approximately equal to the predicted value of $(p)^{-1/2} = 0.353$ (Jolicoeur, 1963) for an isometric size vector. It is therefore reasonable to regard principal component 1 as an indication of size. So 86.8% of the variance in the data set is due to size differences independent of shape. This is to be expected where a range of sizes of limpets are sampled.

Table 3.3 - Eigenvalues for data comparing mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol. Data have been log10(x+1) transformed and a correlation matrix used to standardize variance.

Measured character	Eigenvalue	Percentage of variance	Cumulative percentage
Principal component 1	6.942	86.8	86.8
Principal component 2	0.609	7.6	94.4
Principal component 3	0.253	3.2	97.6
Principal component 4	0.074	0.9	98.5
Principal component 5	0.056	0.7	99.2
Principal component 6	0.029	0.4	99.5
Principal component 7	0.023	0.3	99.8
Principal component 8	0.014	0.2	100.0

Table 3.4 - Eigenvectors for data comparing mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol. Data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize variance. $P^{1/2}$ is the predicted score for each element in principal component 1 if principal component 1 is an isometric size vector.

Measured character	Principal component 1	Principal component 2	Principal component 3	Principal component 4
% of variance	86.8 %	7.6 %	3.2 %	0.9 %
Length	0.363	-0.298	0.102	-0.022
Width (widest)	0.365	-0.301	0.108	-0.277
Height (width)	0.340	0.454	-0.486	-0.151
Height (length)	0.353	0.367	-0.396	0.135
Apex	0.359	-0.282	-0.013	0.811
Width (apex)	0.364	-0.247	0.054	-0.466
Radula	0.308	0.567	0.761	0.057
Cube root of weight	0.372	-0.134	-0.055	-0.071
$P^{1/2}$	0.353			

Table 3.5 - Two way analysis of variance of the four groups (Irepetra mesolittoral, Irepetra infralittoral, Limassol mesolittoral and Limassol infralittoral) for the first four principal components and group means shown where there is a significant result.

				Principal component group mean			
		F-value	*** - P<0.001 ** - P<0.005	Irepetra mesolittoral	Irepetra infralittoral	Limassol mesolittoral	Limassol infralittoral
Component 1	Country	59.57	***	-0.752	1.945	-2.623	1.038
	Zone	207.95	***				
	Interaction	4.64	N.S.				
Component 2	Country	7.05	N.S.				
	Zone	46.58	***	0.279	-0.367	0.360	-0.154
	Interaction	0.62	N.S.				
Component 3	Country	12.65	***	0.065	0.134	-0.102	-0.090
	Zone	0.42	N.S.				
	Interaction	0.24	N.S.				
Component 4	Country	0.08	N.S.				
	Zone	1.38	N.S.				
	Interaction	1.84	N.S.				

Table 3.5 summarises the results of a two-way ANOVA of the first four principal components between shore zone and location. The first principal component (the isometric size vector) shows significant differentiation between both countries and zones. The second principal component differentiates between the two zones whilst the third differentiates between countries. In none of the principal components is there a significant interaction between zone and country, showing these two factors and their effects on shell shape to be acting independently of one another. Even though principal component 2 accounts for 7.6% of the variance it shows no differentiation between limpets from different countries. This highlights a problem with principal component analysis. Since principal component analysis does not include information on the groups in the analysis the resultant axes do not necessarily reflect inter-group variation. The orientation of the principal axes will be determined by intra-group as well as inter-group variance. So although 7.6% of the variance is explained by the second component that variance is not country-specific. Principal component 3 shows significant differences between the two countries.

Principal component 2 shows no single dominant element (Table 3.4). Radula length and the two height measures contribute most to this component, all three showing a positive correlation. The other elements (excluding cube root of weight) contribute to a lesser degree and show a negative correlation. Individuals with a high principal component 2 score will, therefore, tend to be taller and have a longer radula length whilst the base area will be less, than those with a lower score for this component.

Principal component 3 is dominated by a positive radula length element, with a negative contribution from the two height variables. Principal component 4 did not differentiate between the different groups and so will not be considered further.

Where the data set is divided between mesolittoral and infralittoral samples a plot of principal component 1 against principal component 2 shows clear groupings (Fig. 3.6). Accepting principal component 1 as an isometric size vector it is clear that infralittoral individuals tend to be larger than mesolittoral ones. Principal component 2 also showed significant differences between these two groups. The distribution of points and the group means suggest that, relative to an infralittoral limpet of the same isometric size, a mesolittoral limpet would be taller; have a longer radula and a small base area. This result is consistent with the

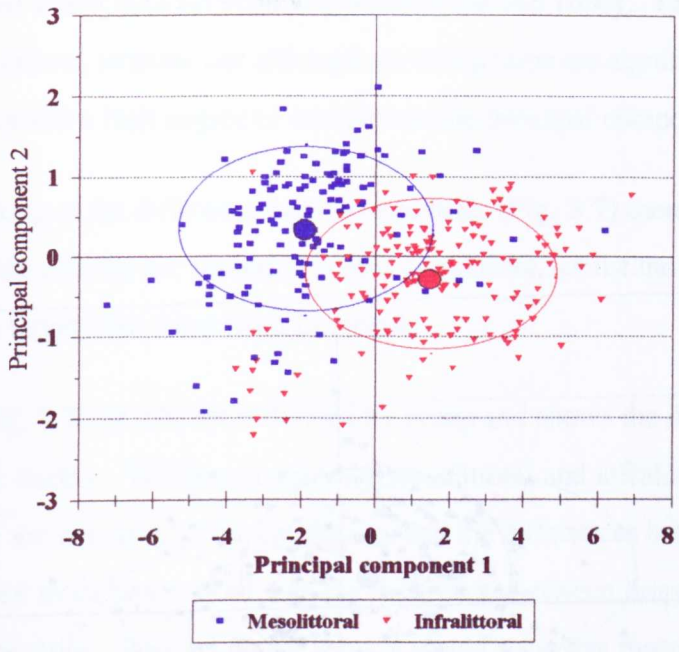


Figure 3.6 - Plot of principal component 1 against principal component 2 for mesolittoral and infralittoral *P.caerulea* from Irepetra and Limassol. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize the data. The data are separated by zone, with group centroids and 95% confidence intervals shown.

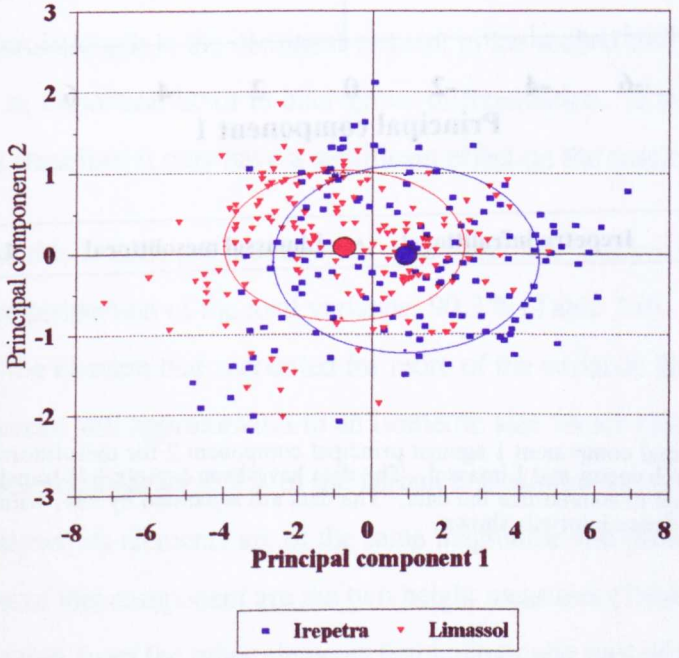


Figure 3.7 - Plot of principal component 1 against principal component 2 for mesolittoral and infralittoral *P.caerulea* from Irepetra and Limassol. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize the data. The data are separated by country, with group centroids and 95% confidence intervals shown.

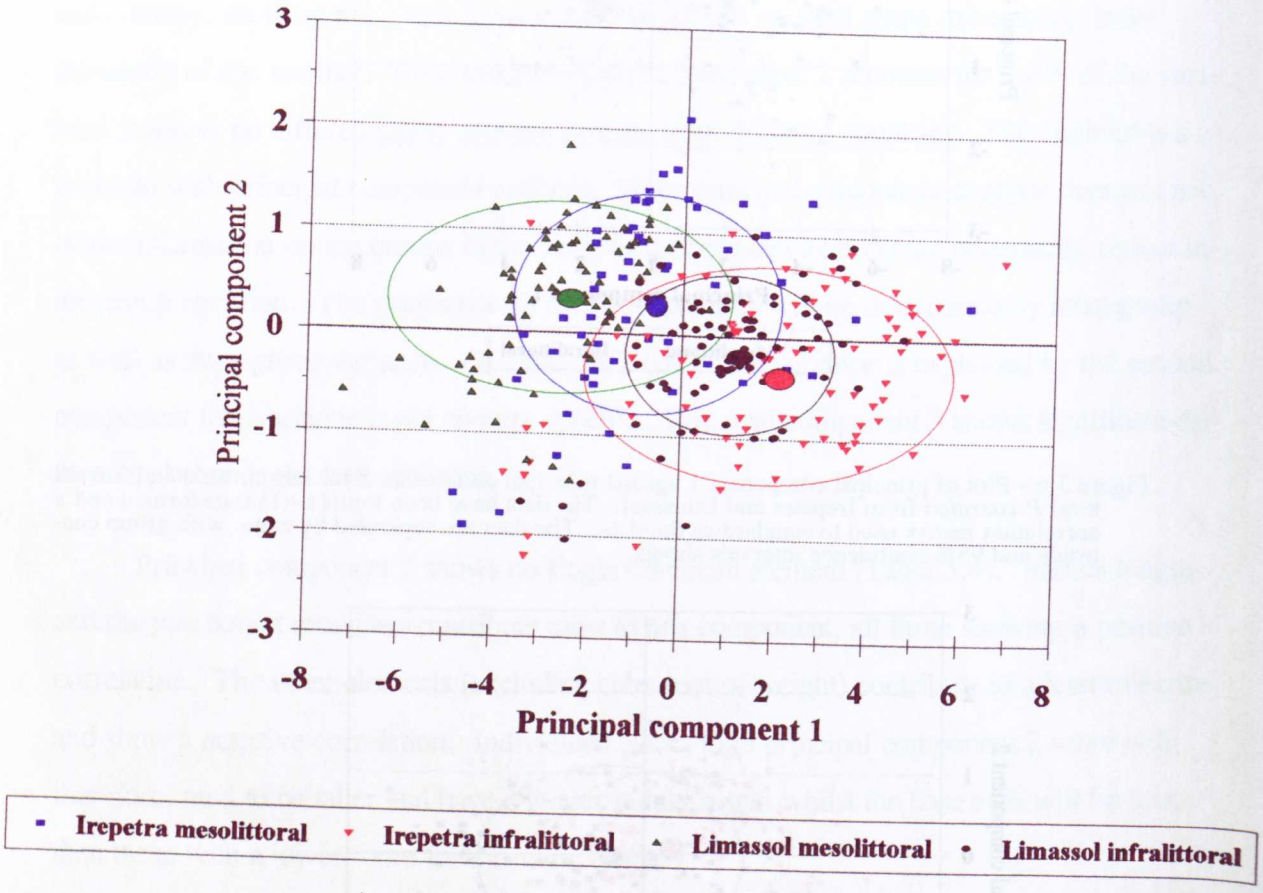


Figure 3.8 - Plot of principal component 1 against principal component 2 for mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize the data. The data are separated by site, with group centroids and 95% confidence intervals shown.

hypothesised differences between limpets from the two zones. The 95% confidence limits of the means shown, indicate that although the two groups are significantly different along both axes there is still a high degree of variation in the principal components within a group.

Looking at the difference between countries (Fig. 3.7) there is no significant separation along the axis for the second principal component, whilst the limpets from Irepetra are on average larger than those from Limassol.

In Fig. 3.8 the data set is divided by group and shows the differences due to zone much more starkly. Within a country the mesolittoral and infralittoral limpets are well separated along the two axes. This emphasises that the differences between mesolittoral and infralittoral are more pronounced than the differences between limpets from the same zone but different countries. Position on the shore it would seem has more effect on the shape of the limpet than does its geographic location.

Radula length is the variable that has the lowest correlation coefficients with other variables (Table 3.2). It will therefore contribute more of the total variance than any other variable. As radula length is the dominant element in the second two principal components it is potentially an important factor in inter-group differentiation. If this element is removed from the analysis therefore it may have a significant effect on the results.

When this is done the first thing of note is that the first principal component accounts for a greater proportion of the total variance, 90.3% (Table 3.6). This is consistent with the removal of the element that accounted for more of the variance than any other. The first principal component still approximates to an isometric size vector (Table 3.7). Principal component 2, which accounts for 6.9% of the variance, is similar to principal component 2 from the first analysis, its elements are of the same magnitude and order of importance. The major elements of this component are the two height measures (Table 3.7) with a lesser, negative contribution from the other elements (excluding cube root of weight). The first two principal components show significant differences between zones and between countries (Table 3.8); whereas in the analysis including radula length the difference between countries was not significant for principal component 2. As before there is no significant interaction term for any of the principal components, showing the two factors to be acting independently. Principal component 3 shows no differences by either factor. In this analysis principal com-

ponent 4 shows signifcant differences by zone (Table 3.7) this component being mainly a contrast of a positive width across apex element against a negative shell length element. In other words infralittoral individuals tend to be narrower across the apex and have a longer shell than mesolittoral individuals.

Table 3.6 - Eigenvalues for data comparing mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol. Data have been log10(x + 1) transformed and a correlation matrix used to standardize variance. Radula length is not included in the analysis.

Measured character	Eigenvalue	Percentage of variance	Cumulative percentage
Principal component 1	6.321	90.3	90.3
Principal component 2	0.482	6.9	97.2
Principal component 3	0.075	1.1	98.3
Principal component 4	0.056	0.8	99.1
Principal component 5	0.029	0.4	99.5
Principal component 6	0.023	0.3	99.8
Principal component 7	0.014	0.2	100.0

Table 3.7 - Eigenvectors for data comparing mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol. Data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize variance. Radula length is not included in the analysis. $P^{1/2}$ is the predicted score for each element in principal component 1 if principal component 1 is an isometric size vector.

Measured character	Principal component 1	Principal component 2	Principal component 3	Principal component 4
% of variance	90.3 %	6.9 %	1.1 %	0.8 %
Length	0.384	-0.287	-0.037	-0.531
Width (widest)	0.386	-0.292	-0.281	-0.042
Height (width)	0.352	0.654	-0.120	0.136
Height (length)	0.366	0.541	0.146	-0.115
Apex	0.338	-0.240	0.821	0.324
Width (apex)	0.385	-0.221	-0.452	-0.646
Cube root of weight	0.392	-0.072	-0.068	-0.402
$P^{1/2}$	0.378			

Table 3.8 - Two way analysis of variance of the four sites for the first four principal components and group means shown where there is a signifcant result. Radula length is not included in the analysis.

				Principal component group mean			
		F-value	*** - P<0.001 ** - P<0.005	Irepetra mesolittoral	Irepetra infralittoral	Limassol mesolittoral	Limassol infralittoral
Component 1	Country	61.13	***	-0.784	1.888	-2.541	1.040
	Zone	229.70	***				
	Interaction	4.70	N.S.				
Component 2	Country	14.63	***	0.178	-0.358	0.325	-0.060
	Zone	36.56	***				
	Interaction	1.00	N.S.				
Component 3	Country	0.35	N.S.				
	Zone	1.48	N.S.				
	Interaction	1.79	N.S.				
Component 4	Country	1.63	N.S.				
	Zone	18.16	***	0.085	-0.024	0.044	-0.077
	Interaction	0.05	N.S.				

One of the first things that is clear from the plots of the principal components (Figs 3.9, 3.10 & 3.11) is that the distribution of the points (and their means) along the first principal component is very similar to the analysis where radula length is included. This lends weight to the assumption that the first component approximates to an isometric size vector (i.e. an index of shell size), as the size vector should be relatively independent of any one of the original variables, since size is determined by the combination of all the variables. Thus if one of the variables were removed the size of the shell is still the same and the combination of the remaining components should be enough to give the same size index.

Where the data are grouped by zone (Fig 3.9) the two means along the principal component 2 axis are closer together, but the tighter grouping along this axis means that there is no loss of discrimination. Mesolittoral limpets tend to be taller relative to their base area than infralittoral limpets of the same size. With radula length not included there is greater inter-country separation in principal component 2 (Fig. 3.10, Table 3.8). Although there is still a large amount of overlap, limpets from Limassol tend to be taller relative to their base

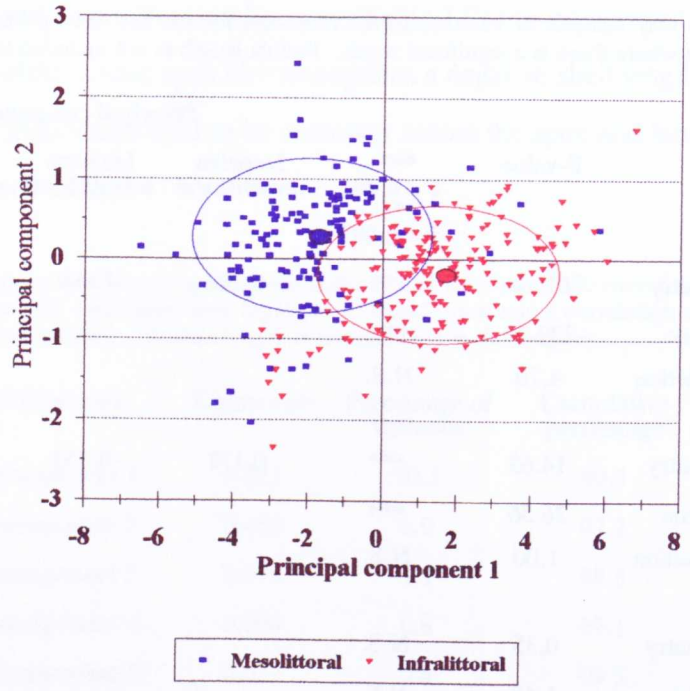


Figure 3.9 - Plot of principal component 1 against principal component 2 for mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize the data. The data are separated by zone, with group centroids and 95% confidence intervals shown. Radula length is not included in the analysis

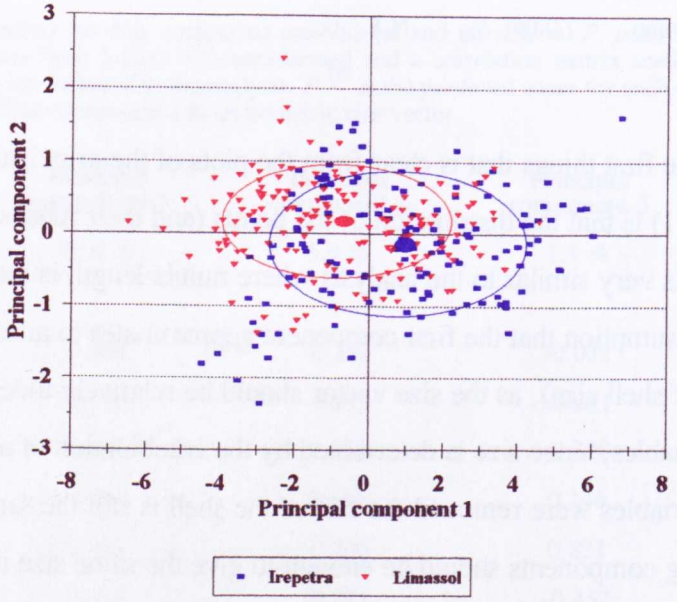


Figure 3.10 - Plot of principal component 1 against principal component 2 for mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize the data. The data are separated by country, with group centroids and 95% confidence intervals shown. Radula length is not included in the analysis.

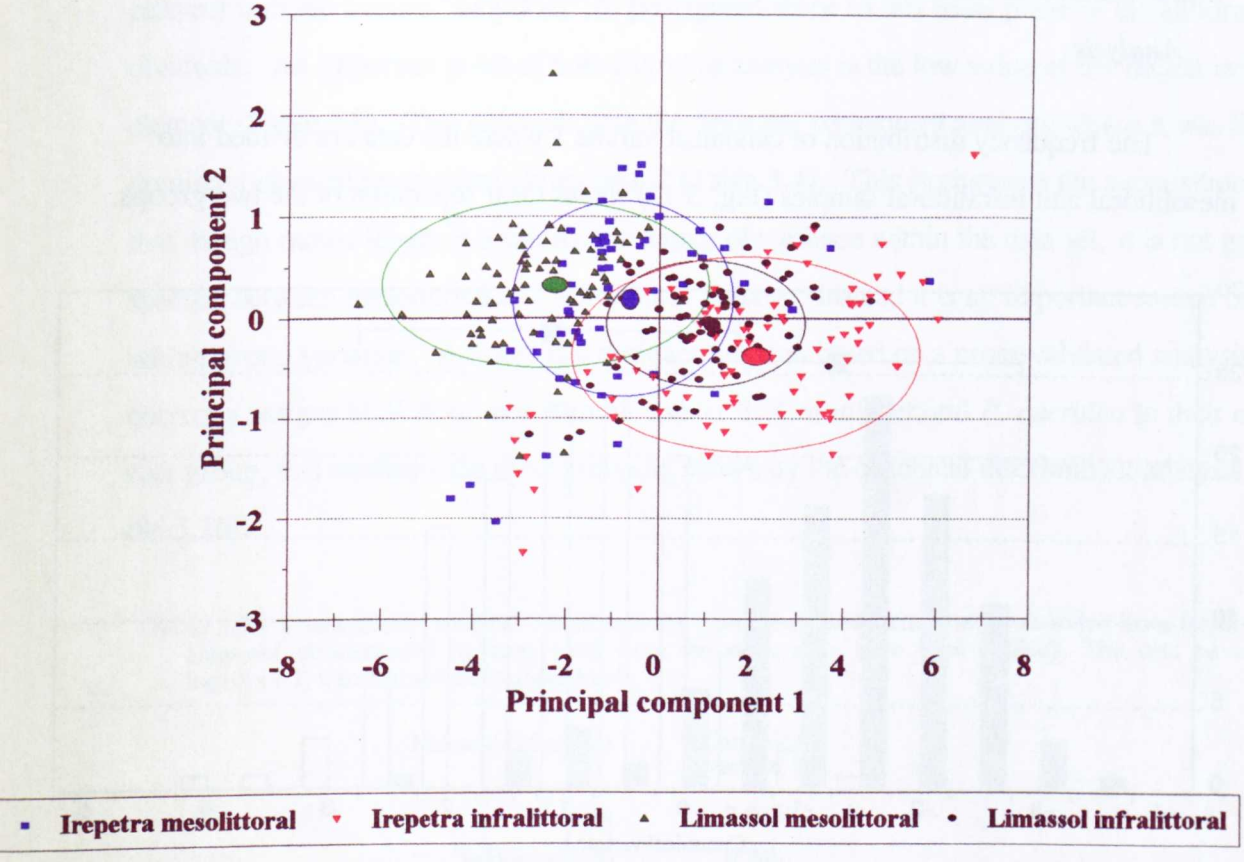


Figure 3.11 - Plot of principal component 1 against principal component 2 for mesolittoral and infralittoral *P.caerulea* from Irepetra and Limassol. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize the data. The data are separated by site, with group centroids and 95% confidence intervals shown. Radula length is not included in the analysis.

area than those from Irepetra. From Fig. 3.11 there does not appear to be a loss of grouping by removing radula length from the analysis. This is probably because although radula length contributes significantly to the variance in the original data set, this variance is not exclusively group-specific. Thus there is a large within-group variation in this character.

3.3.4- Canonical Discriminant Analysis and Cross-validated Discriminant Function Analysis

The frequency distribution of canonical variate 1 where the data are divided into mesolittoral and infralittoral samples (Fig. 3.12) shows clear separation of the two groups.

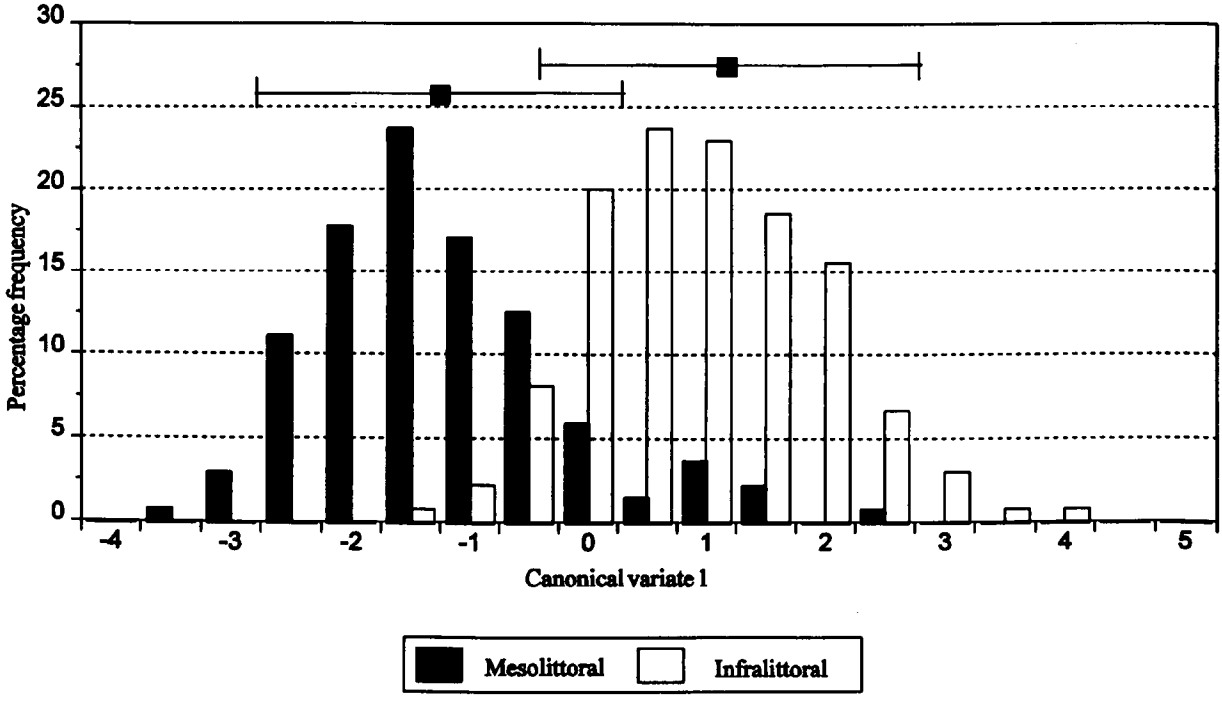


Figure 3.12 - Percentage distribution along canonical variate 1 for mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol differentiated by zone (data from the same zone have been pooled). The data have been log10(x+1) transformed and standardized. The data are separated by zone, with group centroids and 95 % confidence intervals of the populations shown.

Although the separation is not complete, with an overlap of the two 95 % confidence intervals, the group means are significantly different. The canonical variate is the axis showing maximal separation between groups. It is mainly a contrast of a positive shell length element, with a negative 'height with width' element and a positive 'height with length' element. The two width elements (one positive, one negative) also contribute significantly to canonical variate 1 (Table 3.9). The elements contributing to canonical variate 1 indicate that

the major differences between the mesolittoral and infralittoral *P. caerulea* are that the infralittoral limpets are longer and not as high for a given size; the contrast of the two height measures suggests that infralittoral limpets have a greater curvature in the basal plane of the shell. The combination of the length and width elements indicates a larger basal area in infralittoral individuals. The negative 'width across apex' coupled with the maximum width element indicate a more "subplanar" or pentagonal shape to this basal plane in infralittoral individuals. An important point of note from this analysis is the low value of the radula length element (Table 3.9). This contrasts with the principal component analysis where it was the dominant element in principal component 2 (Table 3.4). This strengthens the supposition that though radula length is a significant source of variance within the data set, it is not group-specific between mesolittoral and infralittoral limpets. Instead it is an important source of within-group variation. A linear discriminant function based on a cross-validated analysis correctly assigns 88.9 % of mesolittoral and 92.8 % of infralittoral *P. caerulea* to their correct group, this confirms the clear grouping shown by the canonical discriminant analysis (Table 3.10).

Table 3.9 - Standardized canonical coefficients for mesolittoral and infralittoral P.caerulea from Irepetra and Limassol differentiated by zone (data from the same zone have been pooled). The data have been log10(x+1) transformed and standardized.

Measured character	Canonical variate 1
Length	2.015
Width (widest)	0.749
Height (width)	-1.844
Height (length)	1.639
Apex	-0.177
Width (apex)	-0.776
Radula	-0.327
Cube root of weight	-0.071

Table 3.10 - Results of a linear discriminant function analysis using cross-validation showing the percentage of individuals from each zone correctly assigned to their original groups.

	Mesolittoral	Infralittoral
Mesolittoral	**88.89**	11.11
Infralittoral	7.23	**92.77**
Percentage allocated to group	43.85	56.15
Percentage of original data set	44.85	55.15

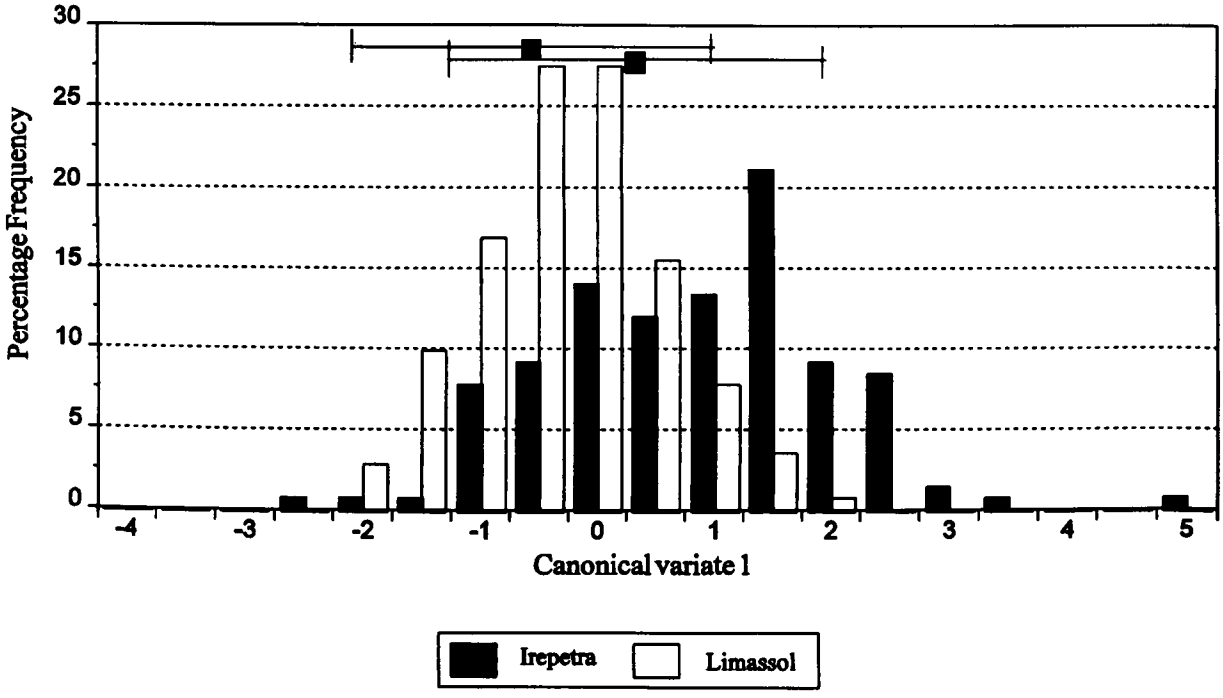


Figure 3.13 - Percentage distribution along canonical variate 1 for mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol differentiated by location (data from the same location have been pooled). The data have been log10(x + 1) transformed and standardized. The data are separated by location, with group centroids and 95% confidence intervals of the populations shown.

Where the data set is divided by country there is much less separation by canonical discriminant analysis (Fig. 3.13). There is more overlap in both the distributions and the 95 % confidence intervals. The overlap of the groups is such that a linear discriminant function will only correctly assign 64 % of the limpets from Irepetra to the correct group (Table 3.12). It is clear from these two methods of analysis that there was not sufficient between-group variation in the shell parameters measured to adequately differentiate between

P. caerulea from Irepetra and Limassol. This is compounded by high within-group variation further masking any differences.

Table 3.11 - Standardized canonical coefficients for mesolittoral and infralittoral *P.caerulea* from Irepetra and Limassol differentiated by location (data from the same location have been pooled). The data have been $\log_{10}(x+1)$ transformed and standardized.

Measured character	Canonical variate 1
Length	-2.185
Width (widest)	0.981
Height (width)	-2.237
Height (length)	1.048
Apex	0.152
Width (apex)	0.966
Radula	0.561
Cube root of weight	1.451

Table 3.12 - Results of a linear discriminant function analysis using cross-validation, showing the percentage of individuals from each location correctly assigned to their original groups (bold).

	Irepetra	Limassol
Irepetra	**64.08**	35.92
Limassol	25.16	74.84
Percentage allocated to group	43.52	56.48
Percentage of original data set	47.18	52.82

Where the limpets are separated into the four sites there is considerable overlap between groups (Fig. 3.14). Though there is some separation of the groups from the same zone along canonical variate 1, this axis mainly acts to divide the mesolittoral from the infralittoral. The first canonical variate has 4 elements significantly contributing to it: there are three positive elements (length, width (max) and height with length) and there is a major negative element (height along width) (Table 3.13). The two height elements again indicate a greater curvature of the basal plane of the infralittoral shells. The length and width elements show infralittoral individuals to be larger, with a greater basal area. The second axis,

accounting for 6.9 % of the variance mainly separates the two infralittoral sites from each other (Fig. 3.14). The important factors in this variate are far from clear with five of the eight elements contributing significant amounts (Table 3.13). Compared with the Limassol infralittoral sample the Irepetra infralittoral individuals were not as long, but are wider and show a greater curvature of the basal plane. The linear discriminant function derived (Table 3.14) further emphasises the split between mesolittoral and infralittoral. For each of the four sites the majority of those individuals misassigned in the cross-validation are assigned to the other site of the same zone. So as was shown in the principal component analysis there is more difference in the shape of limpets between infralittoral and mesolittoral zones, than there is between limpets from widely separated geographic locations. The low correct assignment percentage for Irepetra mesolittoral limpetssuggests that this sample is more variable than the other three.

Table 3.13 - Standardized canonical coefficients for mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol differentiated by zone and location (i.e. four groups: Irepetra mesolittoral, Irepetra infralittoral, Limassol mesolittoral and Limassol infralittoral). The data have been $\log_{10}(x+1)$ transformed and standardized.

Measured character	Canonical variate 1	Canonical variate 2
Percentage of the variance	91.5%	6.9%
Length	1.325	-4.589
Width (widest)	0.971	1.506
Height (width)	2.378	-2.505
Height (length)	1.834	1.263
Apex	-0.077	0.476
Width (apex)	-0.433	0.874
Radula	-0.142	0.671
Cube root of weight	0.338	2.180

An important point made earlier is again borne out for the data divided by site (Table 3.9) and that is the low relative importance of radula length in distinguishing between *P. caerulea* from different zones or countries.

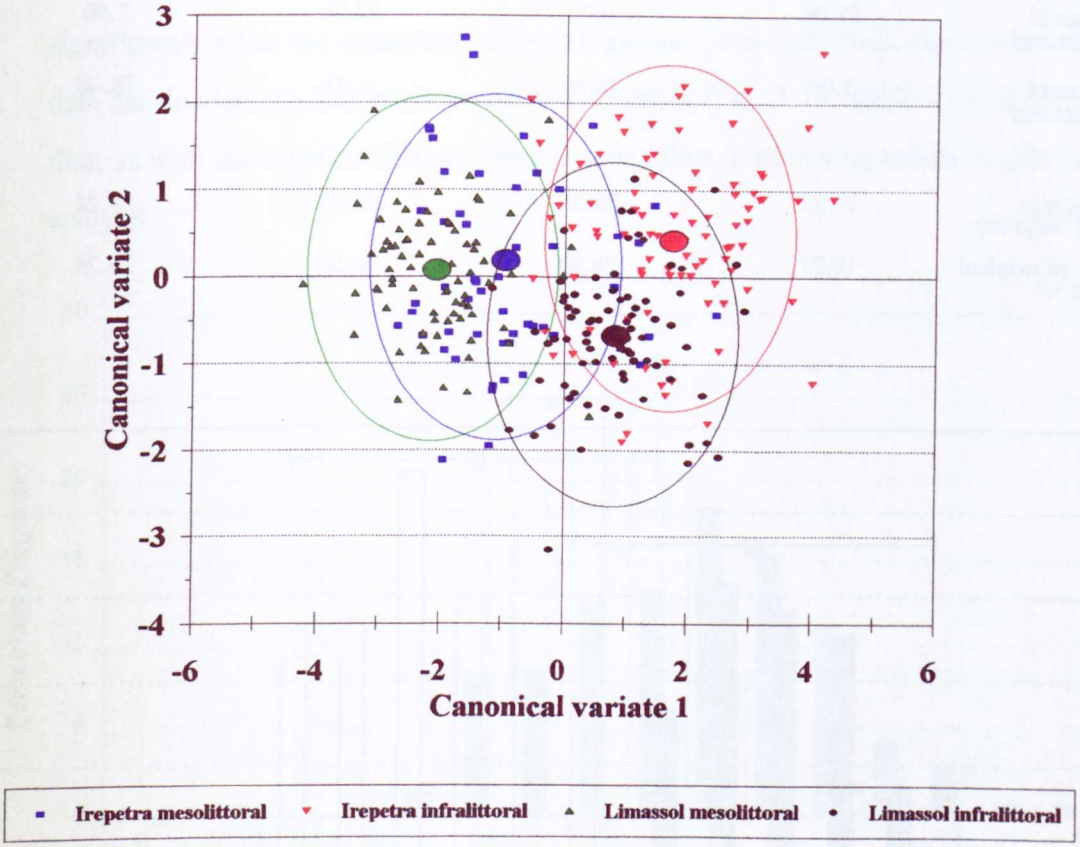


Figure 3.14 - Differentiation of the different groups along canonical variate 1 for the mesolittoral and infralittoral *P.caerulea* from Irepetra and Limassol. The data have been $\log_{10}(x+1)$ transformed and standardized. The data are separated by zone and location, with group centroids and 95% confidence intervals of the population shown.

Table 3.14 - Results of a linear discriminant function analysis using cross-validation, showing the percentage of individuals from each site correctly assigned to their original groups (bold).

	Irepetra mesolittoral	Irepetra infralittoral	Limassol mesolittoral	Limassol infralittoral
Irepetra mesolittoral	**43.64**	9.09	34.55	12.73
Irepetra infralittoral	8.05	**70.11**	2.30	19.54
Limassol mesolittoral	25.00	0.00	**72.50**	2.50
Limassol infralittoral	5.06	16.46	0.00	**78.48**
Percentage allocated to group	18.27	26.25	26.25	29.24
Percentage of original data set	18.27	28.90	26.58	26.25

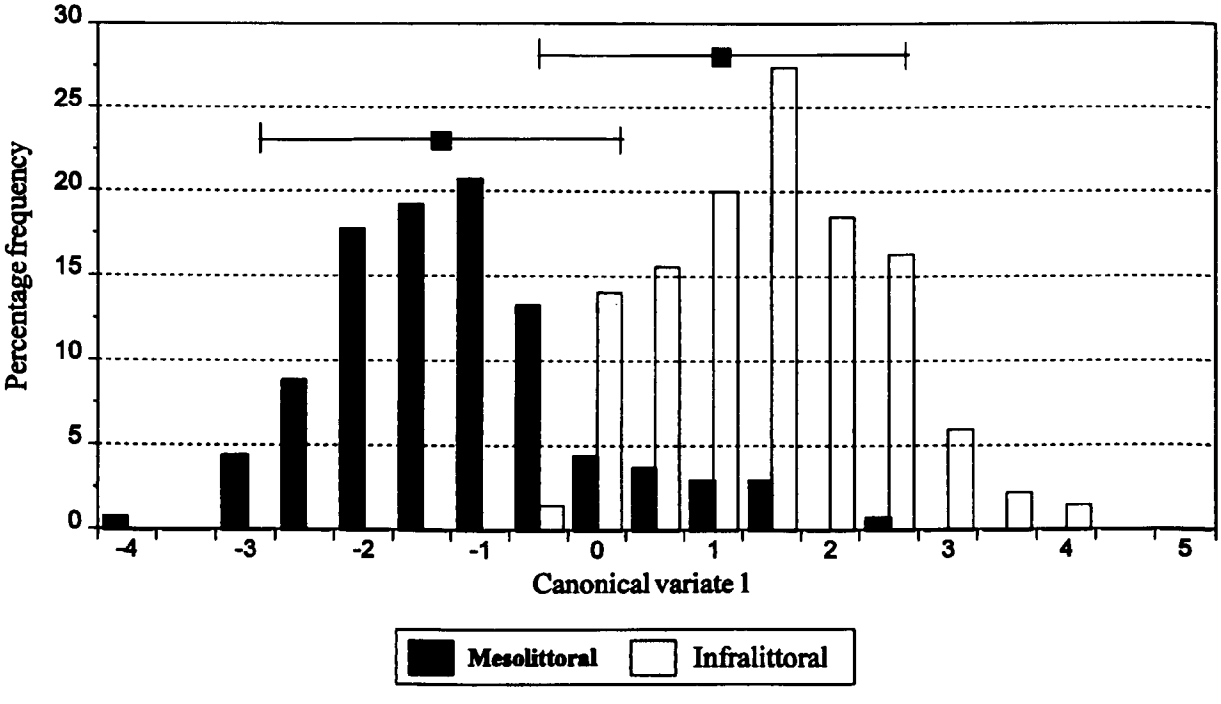


Figure 3.15 - Percentage distribution along canonical variate 1 for mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol differentiated by zone (data from the same zone have been pooled). The data have been log10(x + 1) transformed and standardized. The data are separated by zone, with group centroids and 95% confidence intervals of the populations shown. Radula length is not included in the analysis

With the low importance of radula length in intra-group separation little effect upon the canonical discriminant analysis would be expected when this element is removed from the analysis. Where separated by zone there is a slight change in the distributions of the two groups, but the group means remain virtually unchanged (Fig. 3.15). The magnitude and order of importance of the elements in the standardized canonical coefficient are the same as

they are when radula length is included in the analysis (Table 3.15). The allocation rule has a slightly higher error rate for both mesolittoral and infralittoral *P. caerulea* (Table 3.16) - but again this is only a very small change. It would thus appear that, as expected, there is very little loss of intra-group separation between mesolittoral and infralittoral limpets when radula length is not included in the analysis. This is due to the fact that the majority of the variance contributed by radula length is not group specific and therefore the factor does not significantly affect the separation of the two groups. It is clear from the results where the data are divided into limpets from the two countries (Fig. 3.16, Table 3.18 & Table 3.17) that, as with the zonation data set, there is little effect in removing radula length from the analysis.

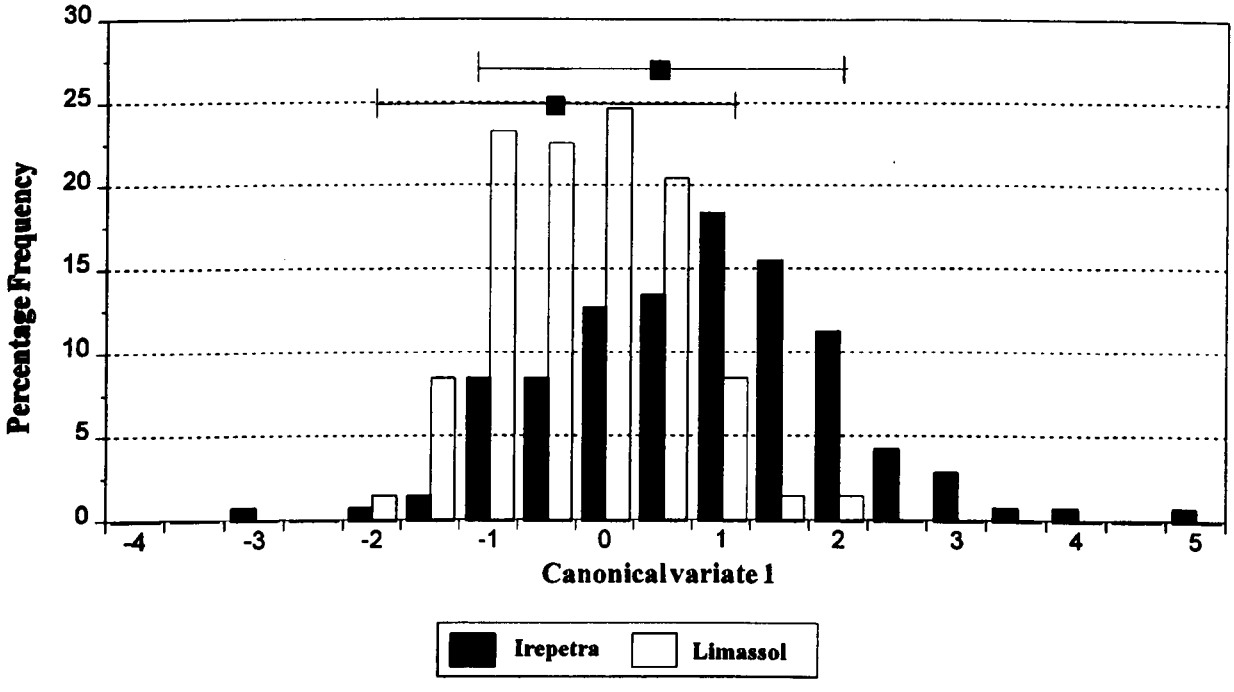


Figure 3.16 - Percentage distribution along canonical variate 1 for mesolittoral and infralittoral *P.caerulea* from Irepetra and Limassol differentiated by location (data from the same location have been pooled). The data have been log10(x + 1) transformed and standardized. The data are separated by location, with group centroids and 95% confidence intervals of the populations shown.

Table 3.15 - Standardized canonical coefficients for mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol differentiated by zone (data from the same zone have been pooled). The data have been $\log_{10}(x+1)$ transformed and standardized. Radula length is not included in the analysis.

Measured character	Canonical variate 1
Length	1.977
Width (widest)	0.447
Height (width)	-1.885
Height (length)	1.439
Apex	-0.045
Width (apex)	-0.786
Cube root of weight	-0.051

Table 3.16 - Results of a linear discriminant function analysis using cross-validation, showing the percentage of individuals from each zone correctly assigned to their original groups. Radula length is not included in the analysis.

	Mesolittoral	Infralittoral
Mesolittoral	**88.15**	11.85
Infralittoral	9.64	**90.36**
Percentage allocated to group	48.89	51.11
Percentage of original data set	44.85	55.15

Table 3.17 - Standardized canonical coefficients for mesolittoral and infralittoral *P.caerulea* from Irepetra and Limassol differentiated by location (data from the same location have been pooled). The data have been $\log_{10}(x+1)$ transformed and standardized. Radula length is not included in the analysis.

Measured character	Canonical variate 1
Length	-2.205
Width (widest)	1.150
Height (width)	-2.219
Height (length)	1.422
Apex	0.029
Width (apex)	1.022
Cube root of weight	1.462

Table 3.18 - Results of a linear discriminant function analysis using cross-validation, showing the percentage of individuals from each location correctly assigned to their original groups (bold). Radula length is not included in the analysis.

	Irepetra	Limassol
Irepetra	**65.49**	34.51
Limassol	28.30	**71.70**
Percentage allocated to each group	45.85	54.15
Percentage of original data set	47.18	52.82

Finally where the data are split by site (Fig. 3.17, Table 3.19 and Table 3.20) there again is virtually no change with radula length removed from the analysis. The group centroids have moved, but only very slightly. The percentage described by each canonical variate is again almost the same with a slight increase to 92.7 % in canonical variate 1 probably due to the reduced intra-group variance. The relative magnitude and order of importance of the elements of the standardized canonical coefficients is the same (Table 3.19). There is some change in the cross-validated linear discriminant function (Table 3.20). Approximately 10 % more of the infralittoral *P. caerulea* from Irepetra are misassigned to Limassol infralittoral. There is an 8 % increase is the numbers misassigned from Limassol infralittoral of which 5 % is to Irepetra mesolittoral. Interestingly there is an increase in the percentage correctly assigned to Limassol mesolittoral. This can be seen as a product of the reduced spread of the data (Fig. 3.17) leaving this as a more separate group.

Although removal of radula length has little effect upon the analysis based on the two zones or on the two countries, there is a noticeable change in the cross-validated linear discriminant function where the data are divided by the four sites. This can be seen to be due to a reduction of the spread of data within groups, as the group means and canonical coefficients are little affected. From Table 3.20 it can be suggested that the change is due to group-specific differences in radula length for the two infralittoral sites. Thus the removal of this element reduces the percentage correctly assigned for these two groups. This does not hold for the mesolittoral groups where there is no effect, and for the mesolittoral *P. caerulea* from Limassol where there is a less discrimination if radula length is included.

Table 3.19 - Standardized canonical coefficients for mesolittoral and infralittoral P.caerulea from Irepetra and Limassol differentiated by zone and location (i.e. four groups: Irepetra mesolittoral, Irepetra infralittoral, Limassol mesolittoral and Limassol infralittoral). The data have been $\log_{10}(x+1)$ transformed and standardized. Radula length is not included in the analysis.

Measured character	Canonical variate 1	Canonical variate 2
Percentage of the variance	92.7%	6.1%
Length	1.279	-4.803
Width (widest)	0.944	1.857
Height (width)	-2.409	-2.648
Height (length)	1.755	1.876
Apex	-0.045	0.395
Width (apex)	-0.426	0.753
Cube root of weight	0.361	2.271

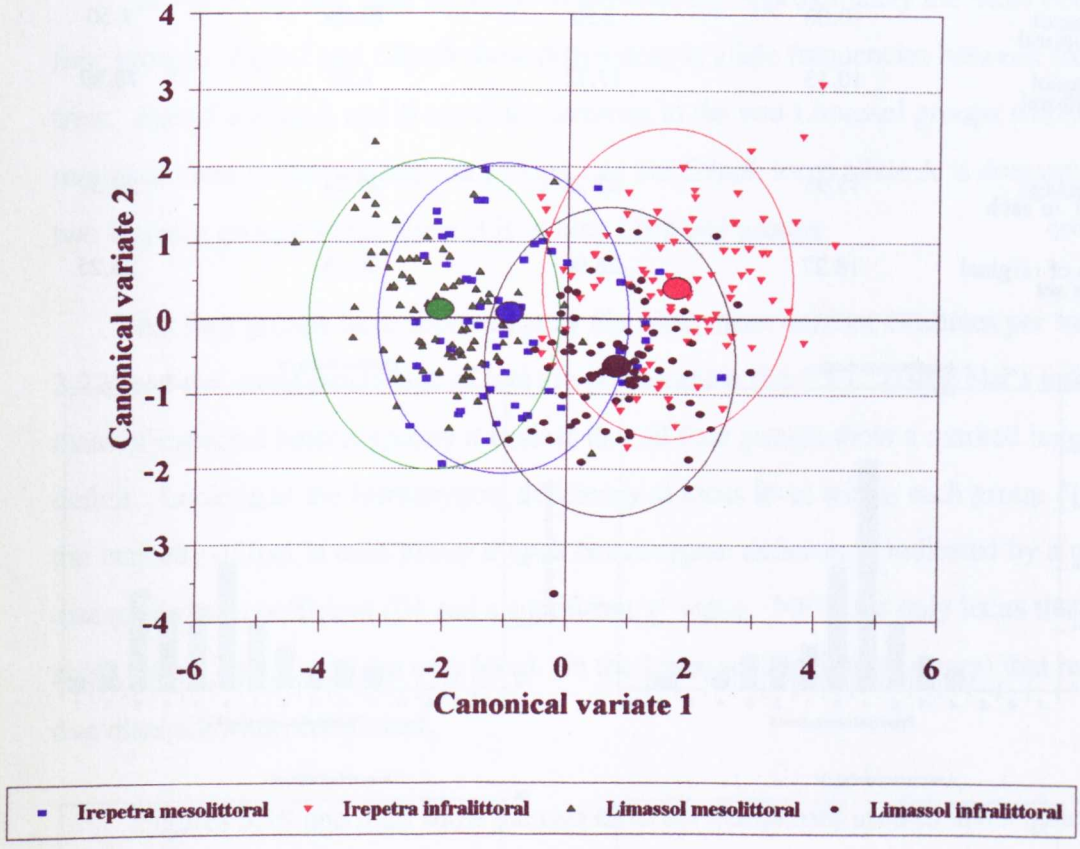


Figure 3.17 - Differentiation of the different groups along canonical variate 1 for the mesolittoral and infralittoral *P.caerulea* from Irepetra and Limassol. The data have been $\log_{10}(x+1)$ transformed and standardized. The data are separated by zone and location, with group centroids and 95% confidence intervals of the population shown. Radula length is not included in the analysis.

Table 3.20 - Results of a linear discriminant function analysis using cross-validation, showing the percentage of individuals from each zone and location correctly assigned to their original groups. Radula length is not included in the analysis.

	Irepetra mesolittoral	Irepetra infralittoral	Limassol mesolittoral	Limassol infralittoral
Irepetra mesolittoral	**43.64**	14.55	32.73	9.09
Irepetra infralittoral	9.20	**59.77**	1.15	29.89
Limassol mesolittoral	10.00	2.50	**85.00**	2.50
Limassol infralittoral	10.13	17.72	1.27	**70.89**
Percentage allocated to each group	15.95	25.25	29.24	29.57
Percentage of original data set	18.27	28.90	26.58	26.25

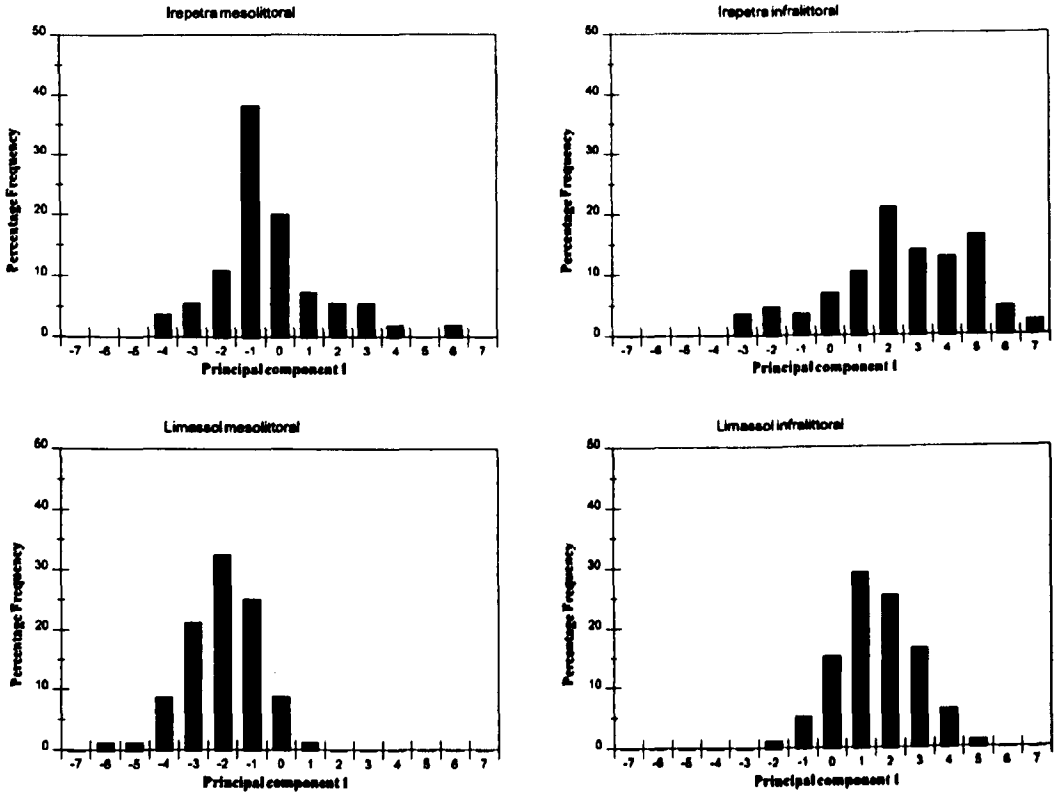


Figure 3.18 - Percentage distribution along principal component 1 for mesolittoral and infralittoral *P. caerulea* from Irepetra and Limassol. With radula length not included in the analysis, this axis acts as an isometric size vector of the shell.

3.3.5 - Electrophoretic analysis.

Of the thirteen loci assayed six were consistently polymorphic (Table 3.21): *Pgi-1*, *Pgm-1*, *G3pdh*, *Pgdh*, *Np* and *Mpi*. The remaining loci were monomorphic and fixed for the same allele in all four groups.

In *Pgi-1*, *Np* and *Mpi* the allele frequencies are approximately the same between the four groups. *Pgm-1* and *G3pdh* show differences in allele frequencies between the two countries. *Pgm-1* alleles A and B are more common in the two Limassol groups whilst allele C is more common in the two Irepetra groups. At the *G3pdh* locus allele A is dominant in the two Irepetra groups whilst allele B is in both Limassol groups.

All four groups have approximately the same mean number of alleles per locus (Table 3.22) and the same percentage of loci are polymorphic (46.2%). Using Nei's unbiased estimate of expected heterozygosity it is clear that all four groups show a marked heterozygote deficit. Looking at the heterozygote deficiency at locus level within each group (Table 3.23), the majority of loci in each group show a heterozygote deficiency, indicated by a negative disequilibrium coefficient (D) and a significant χ^2 value. NP is the only locus that does not show such a deficit, and the only locus (in the Limassol infralittoral group) that has a positive disequilibrium coefficient.

Figures 3.19 and 3.20 show the two different hierarchies used to investigate the interrelations between the four groups. The first hierarchical structure treats the two shore levels as subgroups of the country. The second treats the countries as subgroups of the shore levels. An analysis of heterogeneity of allele frequencies based on the first hierarchical structure (Fig 3.19, Table 3.24) shows that within a site there are no significant differences in allele frequency between the two shore levels. Analysis based on the second hierarchical structure (Fig. 3.20, Table 3.25) shows there to be significant differences in allele frequencies between groups from the same shore level but different countries (when averaged over all six polymorphic loci). Clearly in the case of both mesolittoral and infralittoral shore levels this overall difference is caused by differences at two loci, *Pgm-1* and *G3pdh*. Referring back to the actual allele frequencies (Table 3.21) both these loci show differences in allele frequency between locations. The analysis of heterogeneity of allele frequencies based on the two hierar-

chical structures demonstrate that groups from the same site show a closer allelic frequency match than do the groups from the same shore level but different countries.

Using Nei's unbiased genetic identity (1978) as a measure of relatedness (Table 3.26 and Fig. 3.21) it is clear that all four groups are very closely related with the level of common identity being 0.978 (Fig 3.21). The very high levels of genetic identity are consistent with the four samples being conspecific (Thorpe 1979) and collected in one hydrogeographical region, the levels of identity being indicative of high levels of gene flow between the sample sites. The point of note though is that the groups from the same site are marginally more closely related than groups from the same shore level. This supporting the results from the heterogeneity of allele frequencies analysis but is in marked contrast to the morphometric results, where in terms of morphology the reverse is true. The reasons for the contrasting genetic and morphometric results will be examined in the discussion.

Table 3.21 - Allele frequencies and sample size (N) at thirteen loci for the four groups: Irepetra mesolittoral , Irepetra infralittoral, Limassol mesolittoral and Limassol infralittoral.

Locus	Irepetra mesolittoral	Irepetra infralittoral	Limassol mesolittoral	Limassol infralittoral
Pgi-1				
(Number)	**55**	**79**	**79**	**77**
A	0.364	0.272	0.316	0.299
B	0.600	0.696	0.658	0.662
C	0.036	0.032	0.025	0.039
Pgm-1				
(Number)	**50**	**48**	**66**	**65**
A	0.280	0.313	0.462	0.469
B	0.180	0.198	0.242	0.285
C	0.220	0.313	0.106	0.046
D	0.320	0.177	0.189	0.200
G3pdh				
(Number)	**45**	**84**	**54**	**61**
A	0.244	0.214	0.667	0.639
B	0.756	0.786	0.333	0.361
Pgdh				
(Number)	**46**	**69**	**80**	**77**
A	0.565	0.630	0.619	0.656
B	0.272	0.217	0.231	0.279
C	0.163	0.152	0.150	0.065
Np				
(Number)	**55**	**79**	**79**	**79**
A	0.064	0.051	0.082	0.108
B	0.936	0.924	0.905	0.886
C	0.000	0.000	0.000	0.000
D	0.000	0.025	0.013	0.006
Mpi-1				
(Number)	**49**	**86**	**80**	**79**
A	0.337	0.233	0.225	0.209
B	0.000	0.035	0.000	0.013
C	0.663	0.733	0.769	0.778
D	0.000	0.000	0.006	0.000
Mdh-1				
(Number)	**55**	**87**	**80**	**79**
A	1.000	1.000	1.000	1.000
Mdh-2				
(Number)	**55**	**87**	**80**	**79**
A	1.000	1.000	1.000	1.000
Ald-1				
(Number)	**55**	**87**	**80**	**79**
A	1.000	1.000	1.000	1.000
Idh-1				
(Number)	**55**	**87**	**80**	**79**
A	1.000	1.000	1.000	1.000
Idh-2				
(Number)	**55**	**87**	**80**	**79**
A	1.000	1.000	1.000	1.000
Me-2				
(Number)	**55**	**87**	**80**	**79**
A	1.000	1.000	1.000	1.000
Got				
(Number)	**55**	**87**	**80**	**79**
A	1.000	1.000	1.000	1.000

Table 3.22 - Summary of the genetic variability in the four groups: Irepetra mesolittoral , Irepetra infralittoral, Limassol mesolittoral and Limassol infralittoral. Shown are the mean sample size per locus; the mean number of alleles per locus; percentage of polymorphic loci (Commonest allele does not exceed 95%) and the mean observed and expected (Nei's unbiased estimate, 1978) heterozygosity proportions.

				Mean Heteozygosity	
Population	**Mean Sample size per locus**	**Mean No. of alleles per locus**	**Percentage of loci polymorphic**	**Observed**	**HdyWbg expected**
Irepetra mesolittoral	52.7	1.8	46.2	0.087	0.215
S.E.	± 1.100	± 0.300		± 0.035	± 0.077
Irepetra infralittoral	81.1	1.9	46.2	0.084	0.201
S.E.	± 3.100	± 0.300		± 0.033	± 0.072
Limassol mesolittoral	76.8	1.9	46.2	0.115	0.206
S.E.	± 2.200	±0.300		± 0.049	± 0.071
Limassol infralittoral	76.2	1.9	46.2	0.131	0.204
S.E.	± 1.700	± 0.300		± 0.049	± 0.069

Table 3.23 - Summary statistic of heterozygosity levels for the six polymorphic loci in the four groups: Irepetra mesolittoral, Irepetra infralittoral, Limassol mesolittoral and Limassol infralittoral. Expected heterozygosite is calculated using Nei's unbiased estimate of heterozygosity. The loci fixation index (F) and disequilibrium coefficient (D) are given and the results of a χ^2 test for significant departure from expected heterozygosity level (Where *** = $p<0.0001$; **= $p<0.001$; *=$p<0.05$; N.S. = $p>0.05$).

Locus	Observed hetero-zygotes	Expected hetero-zygotes	Fixation index (F)	D	χ^2	D.F	*p*
Irepetra mesolittoral							
Pgi-1	10	28.11	0.641	-0.644	29.373	3	***
Pgm-1	19	37.29	0.485	-0.491	53.219	6	***
G3pdh	6	16.81	0.639	-0.643	19.345	1	***
Pgdh	13	26.98	0.513	-0.518	24.139	3	***
Np	5	6.62	0.237	-0.244	3.791	1	N.S.
Mpi-1	3	22.11	0.863	-0.864	37.558	1	***
Irepetra infralittoral							
Pgi-1	12	35.00	0.655	-0.657	43.835	3	***
Pgm-1	17	35.61	0.518	-0.523	44.848	6	***
G3pdh	12	28.46	0.576	-0.578	28.767	1	***
Pgdh	19	36.99	0.483	-0.486	43.686	3	***
Np	6	11.36	0.469	-0.472	108.791	3	***
Mpi-1	8	35.30	0.772	-0.773	100.671	3	***
Limassol mesolittoral							
Pgi-1	6	37.05	0.837	-0.838	66.851	3	***
Pgm-1	37	45.26	0.176	-0.182	56.757	6	***
G3pdh	12	24.22	0.500	-0.505	14.078	1	***
Pgdh	29	43.57	0.330	-0.334	28.214	3	***
Np	13	13.83	0.054	-0.060	0.760	3	N.S.
Mpi-1	9	28.85	0.686	-0.688	41.909	3	***
Limassol infralittoral							
Pgi-1	12	36.47	0.669	-0.671	55.288	3	***
Pgm-1	35	43.02	0.180	-0.186	36.600	6	***
G3pdh	18	28.36	0.360	-0.365	8.303	1	***
Pgdh	27	37.80	0.281	-0.286	12.447	3	**
Np	18	16.16	-0.121	0.114	1.227	3	N.S.
Mpi-1	11	27.84	0.602	-0.605	35.125	3	***

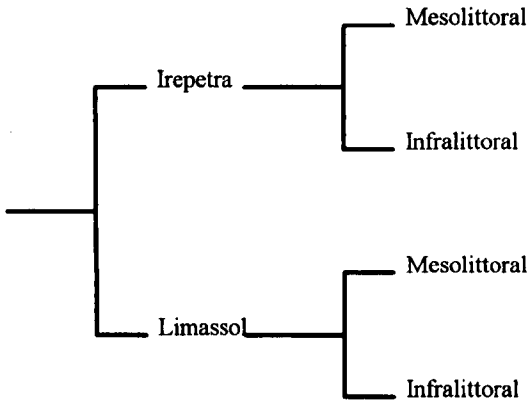


Figure 3.19 - Hierarchical structure used to investigate differences between groups from the same site.

Table 3.24 - Summary of Chi-squared statistics for the analysis of heterogeneity of allele frequencies over six polymorphic loci between the mesolittoral and infralittoral shore levels. Results are shown for both locations, Irepetra and Limassol. Shown are No. of alleles, Chi-squared value, degrees of freedom (D.F.) and probability level (*p*), where *** = $p<0.0001$; **= $p<0.001$; *=$p<0.05$; N.S. = $p>0.05$.

Crete

Locus	No. of alleles	Chi-square	D.F.	*p*
Pgi-1	3	2.709	2	N.S.
Pgm-1	4	5.839	3	N.S.
G3pdh	2	0.306	1	N.S.
Pgdh	3	1.112	2	N.S.
Np	3	2.991	2	N.S.
Mpi-1	3	6.348	2	N.S.
(Totals)		19.306	12	N.S.

Cyprus

Locus	No. of alleles	Chi-square	D.F.	*p*
Pgi-1	3	0.535	2	N.S.
Pgm-1	4	3.567	3	N.S.
G3pdh	2	0.189	1	N.S.
Pgdh	3	6.122	2	N.S.
Np	3	0.898	2	N.S.
Mpi-1	4	3.118	3	N.S.
(Totals)		14.429	13	N.S.

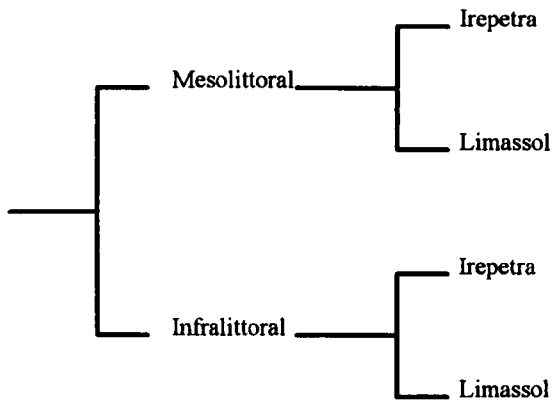


Figure 3.20 - Hierarchical structure used to investigate differences between groups from the same site.

Table 3.25 - Summary of Chi-squared statistics for the analysis of heterogeneity of allele frequencies over six polymorphic loci between the two countries. Results are shown for both shore levels. Shown are No of alleles, Chi-squared value, degrees of freedom (D.F.) and probability level (*p*), where *** = $p<0.0001$; **= $p<0.001$; *=$p<0.05$; N.S. = $p>0.05$.

Mesolittoral

Locus	No. of alleles	Chi-square	D.F.	*P*
Pgi-1	3	1.042	2	N.S.
Pgm-1	4	14.658	3	**
G3pdh	2	35.096	1	***
Pgdh	3	0.733	2	N.S.
Np	3	2.771	2	N.S.
Mpi-1	3	4.378	2	N.S.
(Totals)		58.677	12	***

Infralittoral

Locus	No. of alleles	Chi-square	D.F.	*P*
Pgi-1	3	0.443	2	N.S.
Pgm-1	4	29.789	3	***
G3pdh	2	53.524	1	***
Pgdh	3	6.403	2	N.S.
Np	3	7.187	2	N.S.
Mpi-1	3	2.117	2	N.S.
(Totals)		99.463	12	***

Table 3.26 - Nei (1978) unbiased genetic identity over thirteen loci between the four groups.

Population	Irepetra mesolittoral	Irepetra infralittoral	Limassol mesolittoral	Limassol infralittoral
Irepetra mesolittoral	*			
Irepetra infralittoral	0.999	*		
Limassol mesolittoral	0.980	0.978	*	
Limassol infralittoral	0.980	0.978	1.000	*

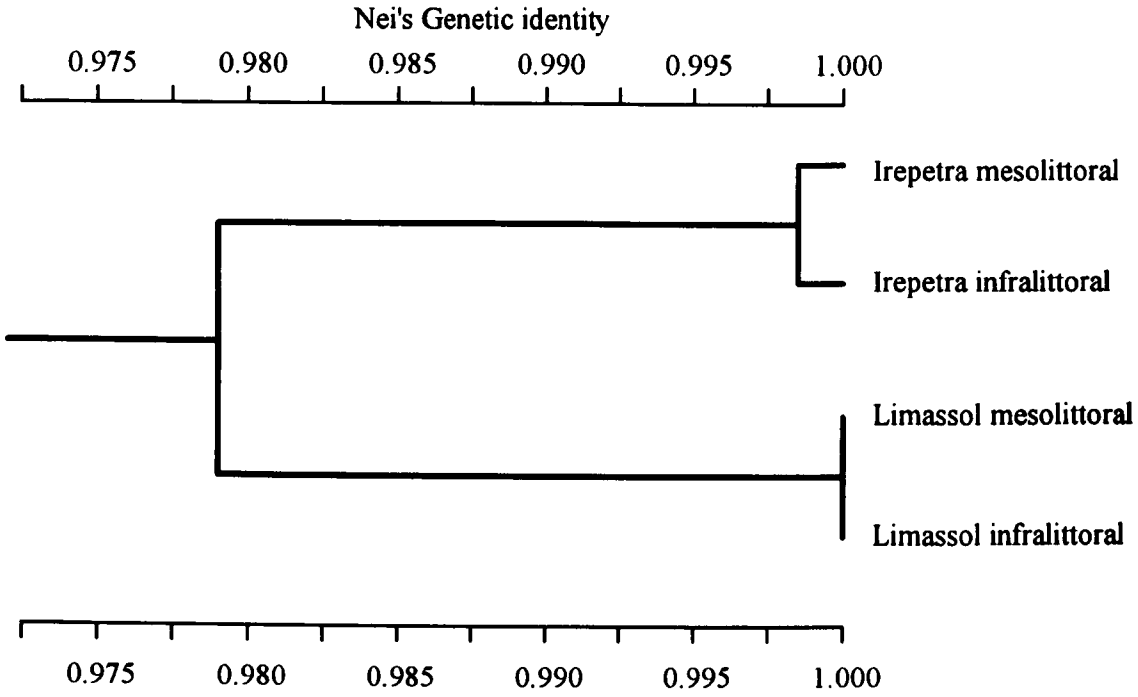


Figure 3.21 - Dendrogram of Nei's (1978) unbiased genetic identity over thirteen loci between the four groups: Irepetra mesolittoral, Irepetra infralittoral, Limassol mesolittoral and Limassol infralittoral.

3.4 - Discussion

3.4.1 - Soft body characters

Patella caerulea is a very variable species with respect to soft body characteristics and shell shape. Even within a sample collected from one shore zone at one site the variation in foot coloration, head tentacle colour and shell shape is considerable. This could be a product of the wide range of microhabitats the species exploits. It appears to be a generalist species that is able to adapt to diverse conditions caused by microhabitat variation.

Some characters seem to be more dominant in individuals from the infralittoral zone. A greater proportion of individuals have an orange foot coloration, translucent pallial tentacles and orange bases to the pallial tentacles. The reasons for these differences are not clear, but it may for be a product of the different microalgal diet of infralittoral individuals as opposed to those in the mesolittoral.

A higher proportion of females in the infralittoral populations is probably because as in many species of limpet (e.g. *P. aspera*), *P. caerulea* starts out as male at first sexual maturity and then some switch to female as they get larger. Therefore as the infralittoral populations comprise larger individuals there is a higher probability of any individual being female. The larger proportion of neuters at Limassol is probably due to the fact that the Irepetra and Limassol samples were collected at different times of the year. The Limassol site being collected during summer - the resting period in the reproductive cycle of *P. caerulea* (Bacci 1947).

3.4.2 - Summary of morphometric results.

From looking at the results of the analysis with and without radula length as a variable it is apparent that there is little effect upon the discriminatory powers of the analysis when the radula length is removed from the analysis. Both principal component analysis and canonical discriminant analysis show tighter grouping in the bivariate plots when radula length is removed, but this reduction in variance is not entirely within-group variance as some discriminatory powers are lost.

It is clear from the results that there is a greater difference in shell size and shape between mesolittoral and infralittoral *P. caerulea* from the same location separated by a few metres than there is between *P. caerulea* from the same zone but widely separated countries. The analysis shows that infralittoral *P. caerulea* are not as tall for a given size as mesolittoral ones. The canonical discriminant analysis suggests that there is a greater curvature to the base area of the shell in infralittoral limpets. Further the two width measures in canonical variate 1 for the zone analysis suggest that infralittoral limpets have a greater maximum width but are not as wide across the apex compared with mesolittoral *P. caerulea* of similar size. That is they display a pentagonal or subplanar shape (Bacci and Sella 1970; Sella and Bacci 1971). This subplanar shape has been described in other work, but only from a subjec-

tive observations (Davies 1969a; Bacci and Sella 1970; Sella and Bacci 1971). This study transforms subjective observations into a quantifiable relationship between measured shell parameters. An important point of note is that the difference between mesolittoral and infralittoral individuals is not discrete (Fig. 3.12 & 3.15) there being approximately 10% overlap in shape (Table 3.15 & 3.23). Note the small second peak in the mesolittoral data distribution along canonical variate 1 in figure 3.12. The subplanar shape is present in the mesolittoral but at a low frequency.

Clearly the differences between countries are less than those within countries. A discriminant function, which maximises the distances between the countries can still only correctly assign 60 - 70 % of the individuals to the correct country. From both principal component analysis and canonical discriminant analysis it would appear that the differences between the two countries are mainly differences in size rather than shape.

The removal of radula length from the numerical analysis is of use in the respect that it allows comparison of the data with historical shell collections where the radula has not been preserved. It also provides a second useful product. The first principal component from an analysis where radula length is not included approximates to an isometric size vector of the shell and thus can be used as a comparative measure of shell size between groups. The greater the principal component 1 value the larger the shell. This has the advantages over a standard univariate measure of size (such as shell length) that were discussed earlier. Mainly it removes the problem of allometric relations between a univariate measure and shell size.

Frequency distributions along principal component 1 (Fig.3.18) show that the infralittoral samples are larger,with respect to shell size, than mesolittoral ones and that Irepetra samples show a higher variation in size than Limassol samples. This difference in size is probably attributable to variation in habitat (Lewis and Bowman 1975) or the low densities of limpets in the infralittoral (Sella and Bacci 1971; Sella 1976) reducing density effects (Lewis and Bowman 1975).

3.4.3 - Genetics versus morphology

The morphometric analysis confirms that, with respect to shape the infralittoral and mesolittoral populations of limpets are two distinct groups. The mesolittoral limpets have an

ovate basal plane. Infralittoral limpets tend to have a subplanar shape and are less high, show greater curvature in the basal plane and have a shorter radula than mesolittoral limpets. There is a degree of overlap in shape (10%) where mesolittoral individuals show shape characteristics associated with infralittoral limpets. Thus from the morphological view point samples from the same shore level but different countries are more similar than samples from the same site but different shore levels. The greater morphological differences between individuals from different shore levels than between individuals from different locations demonstrates, not surprisingly, that the environmental gradient up the shore from infralittoral to mesolittoral is sharper than the geographic environmental gradient from Crete to Cyprus. If morphological data alone was used it might be tempting to call the two populations different species or at least different varieties as has been done in older work (see Christiaens 1973).

The biochemical genetics shows a reverse of this situation. Nei's genetic distance shows that samples from the same site are the most closely related. The analysis of heterogeneity of allele frequencies show no significant differences between allele frequencies from infralittoral and mesolittoral samples taken from the same site. This indicates that there are no barriers to gene flow between the shore levels. That is genetically speaking the two shore levels can be regarded as one population. The genetic distance measures are all close to 1.00 (lowest 0.978) these being consistent with the samples from all four groups being conspecific (Thorpe 1979, 1982). The heterogeneity of allele frequencies found between sites indicates that there is not free gene flow between Irepetra and Limassol.

As has been stated the infralittoral population is only found in patches where there is a break in the algal cover. Accepting that the infralittoral and mesolittoral groups within a site show no barriers to gene flow, yet are morphologically distinct, the source of the infralittoral populations is of interest. There are a number of possible sources:

1) A genetically distinct population that is self-recruiting or recruits from other infralittoral populations.

2) An opportunistic population formed by occasional settlement of *P. caerulea* in clear areas of the infralittoral zone.

3) A population formed by individuals migrating from the mesolittoral zone into clear areas of the infralittoral zone.

This study has not shown any significant genetic differences between infralittoral and mesolittoral groups within a site. This does not necessarily mean that differences do not exist. There could be differences in other loci not included in this study or there is the possibility that there were too few loci studied to reveal genetic differentiation. However the fact that there is genetic differentiation between countries (albeit at a low level) within the polymorphic loci used supports the supposition that there is in fact free gene flow between the two shore levels. The possibility of group specific differences that are undetected by the study is quite high as only thirteen loci were studied and electrophoresis only detects a low proportion of genetic differences (Nei, 1987). If such differences exist this study does suggest that they are not preventing gene flow between shore levels. External fertilisation and a larval dispersal stage will act to prevent the accumulation of genetic differences between habitats. Consistent habitat specific differences will only occur where there is settlement and post-settlement selection acting on each recruitment event. The fact that there is free gene flow between shore levels allows us to reject the option that the infralittoral group is a separate population. Another factor against this option is the lack of juveniles found in the infralittoral zone (C.Nobles *pers obs*). One would expect to find juveniles if settlement were occurring in the infralittoral, for they are present in the mesolittoral zone. It is possible that juveniles are present but very rare.

The second option, opportunistic settlement directly into the infralittoral zone is also unlikely as again the lack of juveniles suggests that settlement does not occur in the infralittoral zone - but it cannot be ruled out.

This leaves the option that the infralittoral population is made up of individuals that have migrated down from the mesolittoral zone. To confirm this a marking or tagging experiment would need to be carried out, but the evidence from this study does seem to support this view, with the lack of juveniles, the larger average size of limpet and the lack of genetic differentiation between zones.

The infralittoral and mesolittoral groups appear to be a single genetic population. In light of this, an 85% separation between the two groups based of shell shape would appear to

be very high. This separation is probably augmented by the bimodal vertical distribution of the species. It is excluded from the infralittoral fringe - the zone between the mesolittoral and the infralittoral - by *P. aspera.* A bimodal distribution will tend to highlight differences between groups as individuals will have to 'choose' one habitat or the other, rather than a position along a cline.

The cline in question is the changing physical conditions from the infralittoral zone to the top of the mesolittoral zone on Mediterranean shores. With the low tidal amplitude and the high air temperatures and irradiance levels experienced when individuals are emersed, the Mediterranean shore represents a very sharp environmental gradient from the infralittoral to the mesolittoral. Thus very different selection pressures are likely to be acting upon limpets in the mesolittoral and infralittoral zones. The mesolittoral limpets will be subjected to a very high desiccation stress and temperature stress, a high spired shape with a small basal area/volume ratio being advantageous in such conditions (Moore 1934; Ebling *et al.* 1962; Davies 1969a). Water movement in the two zones will act differently (Pérès 1967). Predation will probably be by a different suite of organisms, which has been shown to affect shell shape in limpets (Hockey and Branch 1983; Lowell 1986, 1987) and the microalgal food source will be different (Della Santina *et al.* 1992). These and other factors may act to cause or maintain shape differences, giving selective advantage to limpets of a particular shape. I would suggest that the subplanar shape of the infralittoral samples must have some advantage in this zone as it has been repeated shown to occur in low shore limpets (Sella and Bacci 1971; Sella 1976; Beaumont and Wei 1991; Nolan 1991; Côrte-Real *et al.* 1992). The differences in shape between mesolittoral and infralittoral limpets is probably largely due to phenotypic response to two very different environments (Moore 1934).

As well as in the infralittoral the subplanar shape is present at a low frequency in the mesolittoral zone. Assuming the infralittoral population to be one formed by migration from the mesolittoral, it is interesting to speculate on the origins of the subplanar shape in the infralittoral limpets. There are three possible scenarios:

1) Migration into the infralittoral causes a change in the growth pattern of the shell.

2) Only subplanar individuals migrate from the mesolittoral zone.

3) Of those individuals that migrate only the ones with a subplanar shape survive.

There is no evidence from the shapes of the shells of the infralittoral limpets to suggest that there is a marked change in growth pattern. One would expect a step or ridge in the shell at the point where the growth pattern changed if it was a single step migration, as would be suggested by the bimodal distribution of *P. caerulea.* To determine which of the second two options occurs would require a tagging experiment.

Variation in shell shape is often attributed to environmental factors (Moore 1934; Ebling *et al.* 1962; Davies 1969a; Branch and Marsh 1978). It would seem in this study that the subplanar shape is genetically determined or at least has a genetic component as it is present in both habitats whilst the mesolittoral shell shape is not. If there was no genetic component one would not expect to find it in the mesolittoral zone. As stated though despite different shapes being advantageous in different zones, external fertilisation and a larval dispersal act to prevent accumulation of change. Another important point is that the infralittoral zone probably does not provide suitable settlement cues or settlement conditions for limpet larvae and so the larvae can only settle in the mesolittoral and infralittoral fringe. This would mean that the infralittoral habitat could only be exploited by migration from the mesolittoral zone.

Further work on the mesolittoral and infralittoral populations concentrating on tagging individuals would be useful to confirm whether migration occurs and to show which individuals migrate. Work on larval settlement in the Mediterranean would be of interest to see if larvae can only settle in a restricted vertical range on the shore. Larval dispersal and settlement work, and reproductive behaviour would also greatly benefit the understanding of the genetic structure of limpet populations and the generation and maintenance of this bimodal distribution.

4 - Genetics and Morphology of *Patella caerulea* throughout the Mediterranean

4.1 - Introduction

Patella caerulea is considered to occur throughout the Mediterranean, with western limits of Tangiers in Morocco and Tarifa in Spain (Fischer-Piette and Gaillard 1959) and an eastern limit in Israel (Lavie *et al.* 1987). Within this range *P. caerulea* displays a very wide variation in soft body characters, shell coloration, shell shape (Christiaens 1973) and radula structure (See Chapter 1 for more details). There is said to be a high level of intraspecific variation in shell shape at any location (Sella 1976; Sella and Bacci 1971), particularly differences between individuals from mesolittoral and infralittoral shore levels (Sella 1976; Sella and Bacci 1971).

Although much work has shown there to be high levels of local variation in the shell shape of *P. caerulea* (Davies 1969; Bacci and Sella 1970; Sella and Bacci 1971; Bannister 1975; Sella 1976), there has been no work on geographical differences. Thus it is not currently known whether there are specific shell forms determined by regional climatic conditions or brought about by barriers to gene flow.

The levels of gene flow in *P. caerulea* have been investigated for sites around Italy (Sella *et al.* 1985). This work indicated high levels of gene flow between Tyrrhenian and Adriatic populations. Further work (Badino *et al.* 1986) extended the study to include sites in France and one site in Corsica. This again showed high apparent levels of gene flow, accounted for by the larval dispersal of *P. caerulea*. An extension of work to cover a larger geographic range would determine whether hydrographical conditions in the Mediterranean restricted this naturally high gene flow between regions, particularly between the Eastern and Western Mediterranean basins.

The aim of this work is to investigate the genetic and morphological variation of *P. caerulea,* throughout most of its geographical range . Current multivariate techniques (Janson and Sundberg 1983a, 1983b; Sundberg 1988a, 1988b; Furman 1990; Mc Donald *et al.* 1991) will be used to examine differences in shell size and shape between the thirteen

samples taken from throughout the Mediterranean. These methods will also be employed to look for regional variation in shell shape characteristics. Biochemical genetics will be used to establish the degree of gene flow in *P. caerulea* throughout its geographical range. Agreements between genetic and morphological analysis will give information relevant to understanding the effect of gene flow levels on morphological characteristics and indicate the extent of regionally dictated variation in shell shape.

4.2 - Materials and methods

4.2.1 - Collection of samples

Samples of limpets were collected from thirteen sites throughout the Mediterranean (See figures 2.1 & 2.2). At each site a sample of approximately 100 individuals was taken in the mesolittoral zone from above the red algal turf zone that marks the top of the infralittoral fringe. Samples from Calvi, El Arenal, Genoa, Irepetra, Isle Rousse, Larnaca, Limassol, Porto Petro and Sitia were all collected on breakwaters (See Chapter2, Section 2 for more details of collections).

4.2.2 - Qualitative soft body characters

A number of soft body characteristics were recorded for each individual: colour of head tentacles, colour of pallial tentacles, presence of orange base to pallial tentacles, and colour of the foot muscle. The sex, gonad stage and colour of each limpet was also recorded (Orton *et al.* 1956).

4.2.3 - Morphological analysis of shell

For each individual a number of shell parameters were measured, to the nearest 0.5mm, using vernier calipers (Figure 2.3): shell length, maximum shell width, shell width as measured across the apex, shell height (with the length of the shell as the baseline), shell height (with the width of the shell as the baseline) and anterior to apex distance. The two shell width measurements give an impression of how ovate the shell is. Differences between the two shell height measures indicate curvature in the basal plane of the shell. Additionally the shell was weighed to the nearest 0.01g and the radula length measured to the nearest 0.5mm. Shells were labelled and stored for future reference.

The thirteen samples from different sites (El Arenal, Mal Pas, Porto Petro, St Tropez, Genoa, Ghallis Point, Xghajra, Calvi, Isle Rousse, Irepetra, Sitia, Larnaca and Limassol) were used in the analysis of the data. The three major techniques used in the analysis of the data were: principal component analysis, canonical variate analysis and linear discriminant function analysis. Further details of these techniques are given in chapter 2.

4.2.4 - Electrophoresis

Electrophoresis on the samples was carried out following the methods described in detail in the general materials and methods (Chapter 2). In brief, for the electrophoretic work the tissue used was the foot muscle of the limpet. The muscle was removed from live limpets; only healthy individuals were sampled. The genetics of the samples of limpets was examined using standard horizontal starch gel electrophoresis (following Selander *et al.* 1971; Harris and Hopkinson 1977; Ferguson 1980). Tris citrate pH 8.0 (Ward and Breadmore 1977) was used as the gel buffer. The gel slices were stained using standard solutions (Shaw and Prasad, 1970; Schaal and Anderson, 1974; Harris and Hopkinson, 1978). Thirteen loci were assayed : *Pgi-1*, *Pgm-1*, *G3pdh*, *Pgdh*, *Np*, *Mpi-1*, *Mdh-1*, *Mdh-2*, *Ald-1*, *Idh-1*, *Idh-2*, *Me-2* and *Got*.

The analysis of electrophoretic results was based on the genotype frequencies of the loci for each species at each site. Genetic statistics were calculated using the program BIOSYS-1 Release 1.7 (Swofford and Selander, 1989).

4.3 - Results

4.3.1 - Soft body characteristics

There is a wide range of coloration of the base of the foot in *P. caerulea* from all thirteen samples (Fig 4.1). The foot generally varies in colour between a light cream and a very dark grey/black. The data used to construct figure 4.1 are based on the dominant colour of the foot of each individual. Generally the central region of the foot is a lighter colour than the outer edge, the centre varying between a light cream and a dark orange. There is no obvious pattern of foot coloration specific to any country or region.

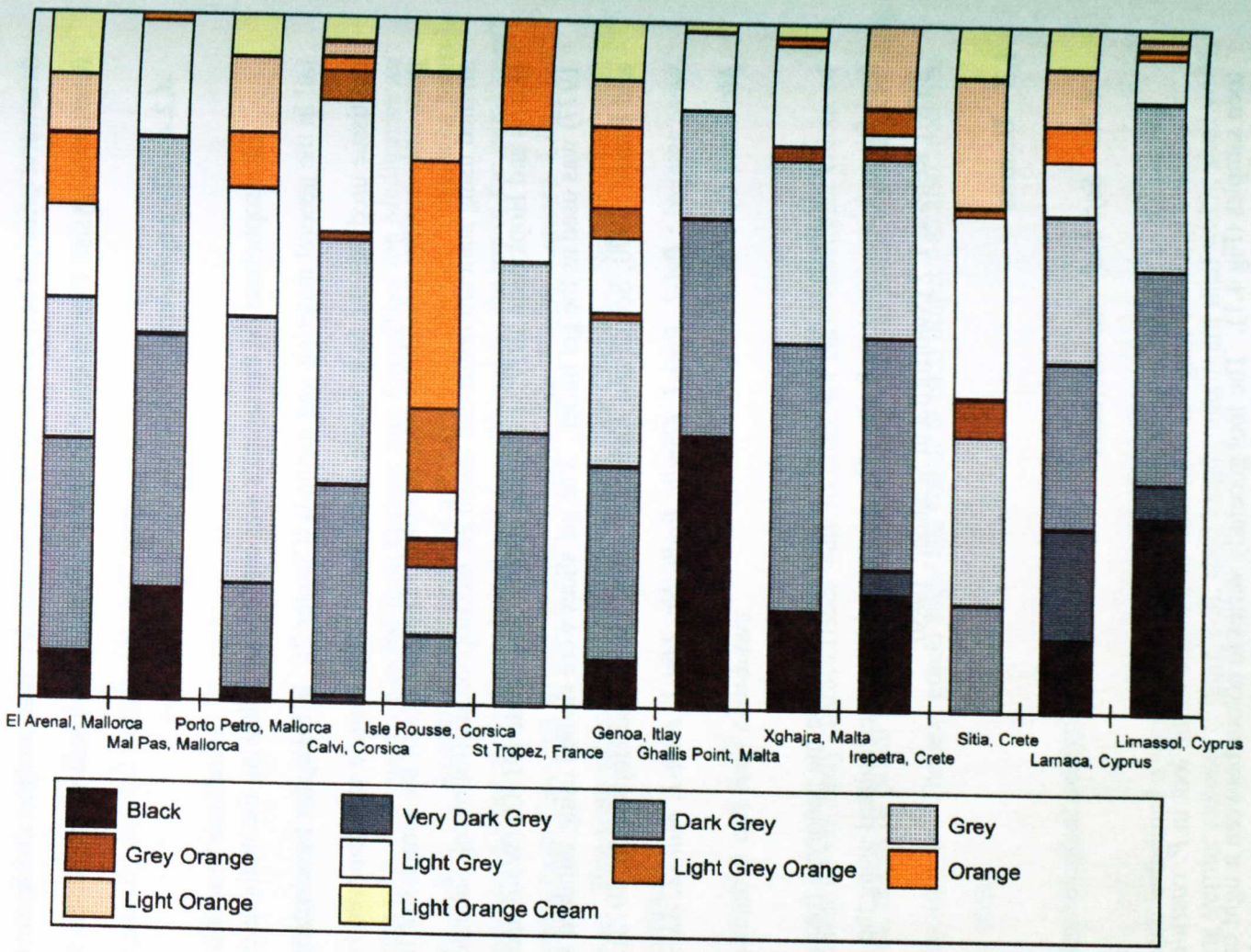


Figure 4.1 - The proportion of limpets of different foot colour for the thirteen samples of *P. caerulea* from the Mediterranean. Proportions given as percentage of the sample. Sample sizes: El Arenal = 153, Mal Pas = 110, Porto Petro = 110, Calvi = 97, Isle Rousse = 99, St Tropez = 20, Genoa = 117, Ghallis Point = 100, Xghajra = 199, Irepetra = 55, Sitia = 99, Larnaca = 37, Limassol = 80.

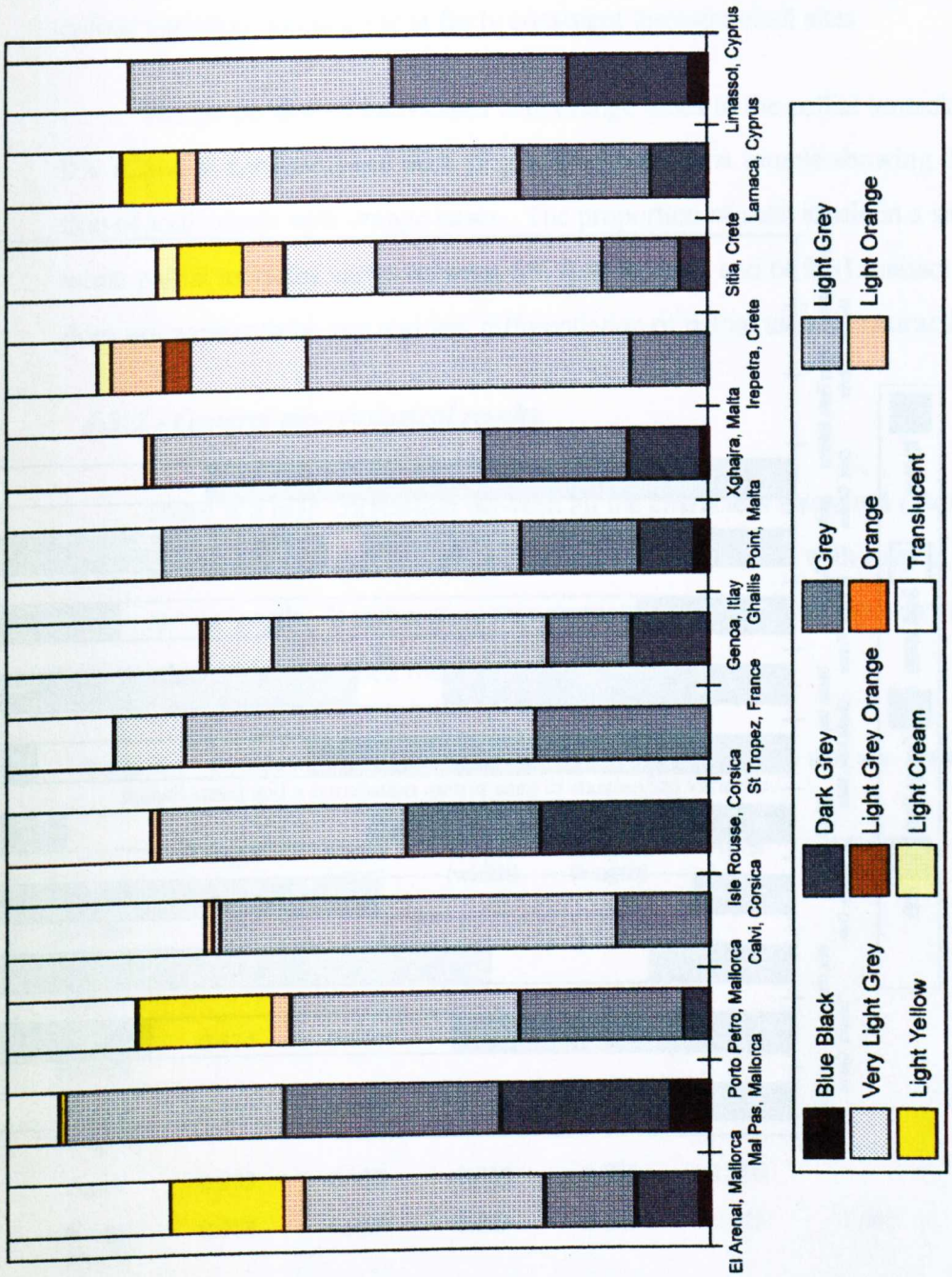


Figure 4.2 - The proportion of limpets of different head tentacle colour for the thirteen samples of *P. caerulea* from the Mediterranean. Proportions given as percentage of the sample. Sample sizes:El Arenal=153, Mal Pas=110, Porto Petro=110, Calvi=97, Isle Rousse=99, St Tropez=20, Genoa=117, Ghallis Point=100, Xghajra=199, Irepetra=55, Sitia=99, Larnaca=37, Limassol=80.

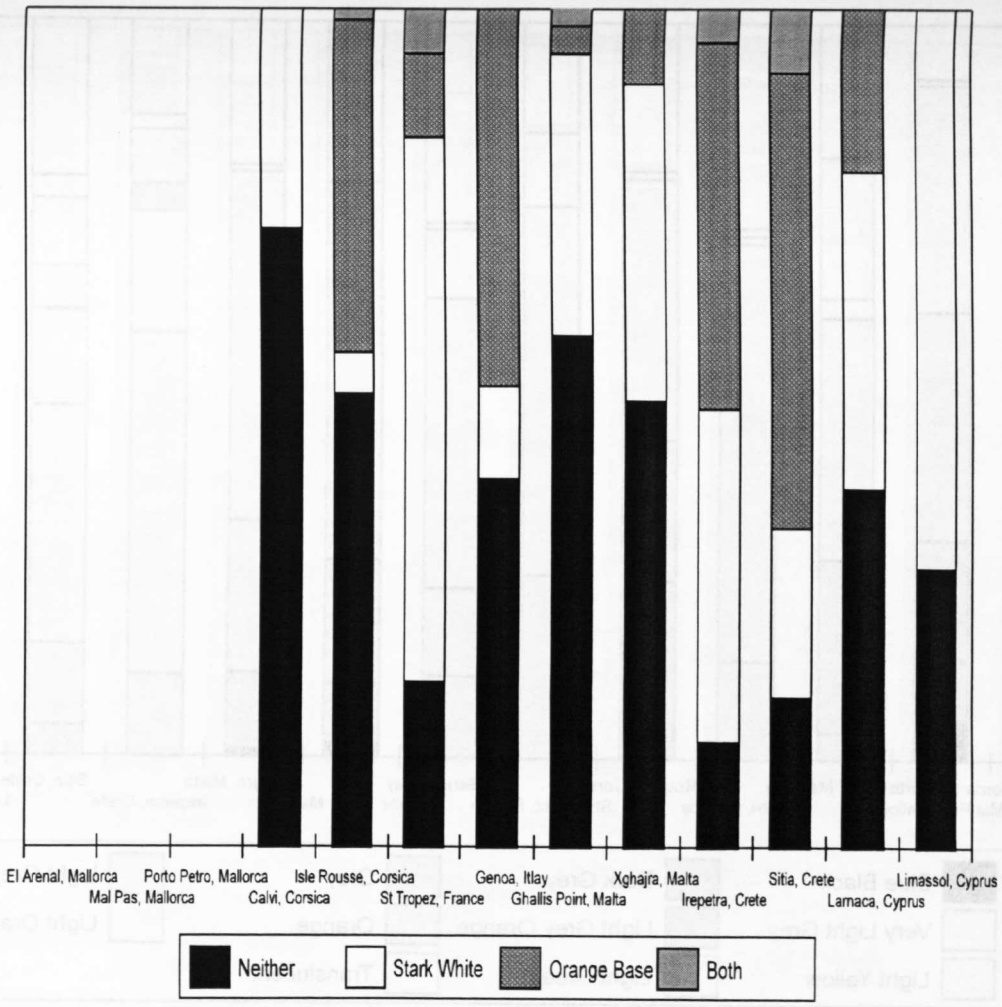


Figure 4.3 - The proportion of limpets of different pallial tentacle colour for the thirteen samples of *P. caerulea* from the Mediterranean. Proportions given as percentage of the sample. There are no results for the Mallorcan samples as these were the first collected and these characters were not scored. Sample sizes: El Arenal = 153, Mal Pas = 110, Porto Petro = 110, Calvi = 97, Isle Rousse = 99, St Tropez = 20, Genoa = 117, Ghallis Point = 100, Xghajra = 199, Irepetra = 55, Sitia = 99, Larnaca = 37, Limassol = 80.

The colour of the head tentacles of *P. caerulea* varies from a dark grey/black through grey to translucent (Fig 4.2). Grey to light grey is the dominant colour in all samples and at most sites approximately 20% of individuals have translucent head tentacles. The range of colour variation within a site is fairly consistent throughout all sites.

The proportion of individuals with orange bases to the pallial tentacles varies between 0% (Calvi & Limassol) and 60% (Fig 4.3) with the Sitia sample showing the highest proportion of individuals with orange bases. The proportion of individuals in a sample with stark white pallial tentacles varies between 6% (Isle Rousse) and 66% (Limassol sample). There does not appear to be any regional differentiation of pallial tentacle characteristic.

4.3.2 - General morphological results.

There is a high correlation between all the characters measured (Table 4.1). The character that consistently shows the lowest correlation coefficient with respect to other characters is radula length. It is therefore this character that shows the greatest variance in the relationship with other shell measures.

Table 4.1 - Correlation coefficients between variables for the complete data set. Data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize variance.

	Length	Width (max)	Height (width)	Height (length)	Apex	Width (apex)	Radula length	Cube root of weigth
Length	1.000							
Width (max)	0.972	1.000						
Height (width)	0.834	0.850	1.000					
Height (length)	0.852	0.847	0.976	1.000				
Apex	0.933	0.922	0.830	0.856	1.000			
Width (apex)	0.958	0.987	0.865	0.862	0.926	1.000		
Radula	0.775	0.778	0.744	0.757	0.718	0.774	1.000	
Cube root of weight	0.942	0.936	0.862	0.881	0.893	0.930	0.791	1.000

Table 4.2 - Eigenvalues for data comparing *P. caerulea* samples from thirteen sites throughout the Mediterranean. Data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize variance.

	Eigenvalue	Percentage of variance	Cumulative percentage

Component 1	7.077	88.5	88.5
Component 2	0.352	4.4	92.9
Component 3	0.324	4.0	96.9
Component 4	0.102	1.3	98.2
Component 5	0.082	1.0	99.2
Component 6	0.037	0.5	99.7
Component 7	0.017	0.2	99.9
Component 8	0.010	0.1	100.0

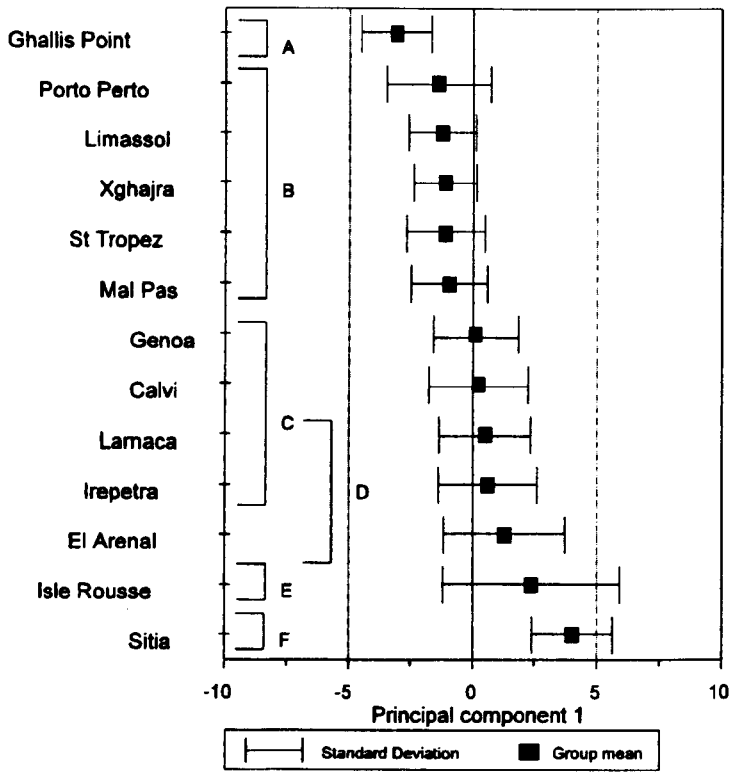


Figure 4.4 - Distribution of the group means of the thirteen samples of *P. caerulea* along principal component 1, with standard deviations shown. There are significant differences between groups ($F=87.99$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log10(x+1)$ transformed and a correlation matrix used to standardise the data.

4.3.3 - Principal component analysis

The first principal component accounts for 88.5% of the variance in the transformed data set (Table 4.2). All the elements of the eigenvector of principal component 1 are approximately equal to each other (Table 4.3) and to the predicted value of $(p)^{-1/2}$ =0.353 for an isometric size vector (Jolicoeur, 1963). It is therefore reasonable to regard principal component 1 as an indication of size, and thus 88% of the variance in the data set (within and between groups) is due to size independent of shape.

Table 4.3 - Eigenvectors for data comparing *P. caerulea* samples from thirteen sites throughout the Mediterranean. Data have been log10(x+1) transformed and a correlation matrix used to standardize variance. $p^{-1/2}$ is the predicted score for each element in principal component 1 if principal component 1 is an isometric size vector.

	Principal component 1	Principal component 2	Principal component 3	Principal component 4
% of variance	88.5 %	4.4 %	4.0 %	1.3 %
Length	0.364	-0.286	0.151	-0.069
Width (max)	0.365	-0.280	0.146	-0.227
Height (width)	0.348	0.346	-0.521	-0.096
Height (length)	0.351	0.331	-0.485	0.079
Apex	0.355	-0.327	-0.046	0.813
Width (apex)	0.366	-0.248	0.081	-0.163
Radula	0.315	0.659	0.661	0.166
Cube root of weight	0.362	-0.086	0.060	-0.466
$p^{-1/2}$	0.353			

Figure 4.4 shows the distribution of sample group means along principal component 1. Clearly the average size of individuals varied between groups. The results of ANOVA and SNK tests shown down the left-hand side of the graph indicate which of these differences in mean size were significant. The Ghallis Point sample had the smallest mean size, whilst the two samples with the largest average size were Isle Rousse and Sitia. If one refers to the basic statistics for this data set it can be seen that the Sitia sample has the highest mean values for each of the elements and the Ghallis Point sample the lowest. This supports the supposition that principal component 1 is an isometric size vector. As can be seen from Figure 4.4 there is considerable overlap between the distributions of the different groups, no group has

a distribution completely separate from the others. There is a graduation of size ranges that does not appear to be related to the geographical location of the sample sites.

Principal component 2 accounts for 4.4% of the variance in the data set (Table 4.3). The dominant element in the eigenvector is a positive radula length element, all other elements except for weight contribute an approximately equal amount to the component. Individuals with a high principal component 2 (e.g., Genoa sample) will tend to have a longer radula relative to the basal area of the shell than individuals with a lower score (e.g., Calvi sample). As is the case for principal component 1 the distribution of sample means along principal component 2 does not appear to relate to country or region of origin (Figure 4.5).

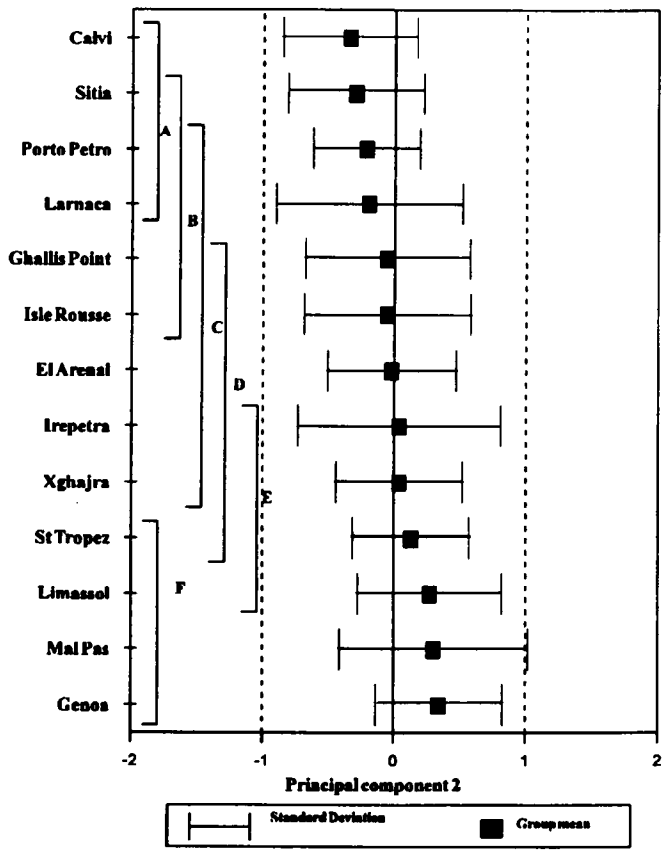


Figure 4.5 - Distribution of the group means of the thirteen samples of *P. caerulea* along principal component 2, with standard deviations shown. There are significant differences between groups ($F=15.62$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log10(x+1)$ transformed and a correlation matrix used to standardise the data.

The ANOVA and SNK test show there to be a considerable degree of overlap between all groups for this component. The only samples from close geographic proximity that fall into the same group in the ANOVA and SNK tests are St Tropez and Genoa, both samples hav-

ing a high group mean along this axis. No sample is clearly differentiated from the others by the SNK test.

The third principal component (4.0% of variance) has an eigenvector strongly dominated by three characters: radula length and the two shell height measures (Table 4.3). The negative correlation between radula length and shell height contrasts with that in principal component 2. Individuals with a high principal component 3 (e.g. Isle Rousse sample) will tend to have a longer radula relative to shell height than individuals with a low score (e.g. Porto Petro sample). As with principal component 2 there is considerable overlap in the distributions of groups, as evidenced by the standard deviations and SNK groupings (Fig 4.6).

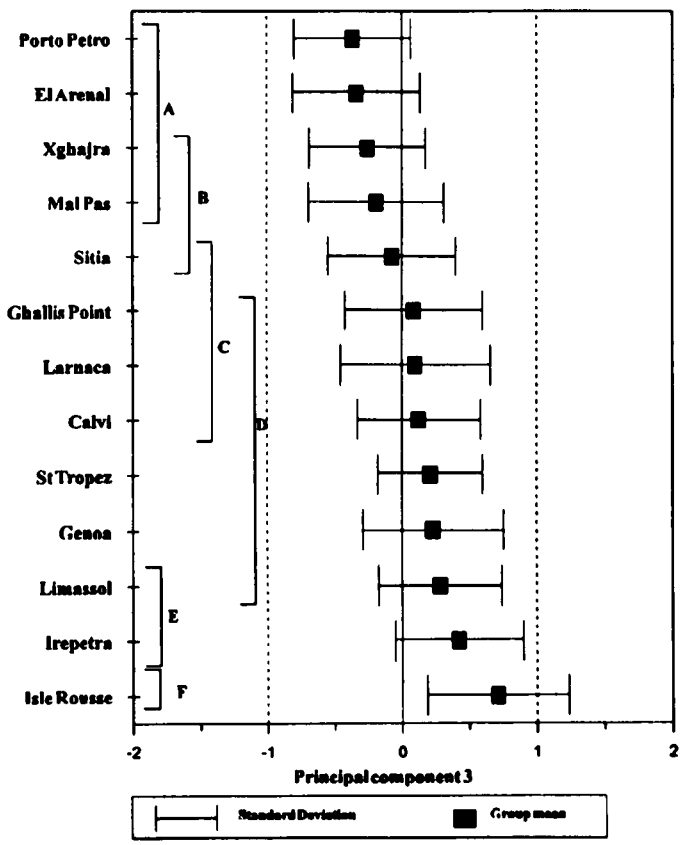


Figure 4.6 - Distribution of the group means of the thirteen samples of *P. caerulea* along principal component 3, with standard deviations shown. There are significant differences between groups ($F=45.95$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log10(x+1)$ transformed and a correlation matrix used to standardise the data.

This principal component does show some regional groupings. As with principal component 2 the Genoa and St Tropez samples have very similar group means. Additionally for this principal component the three sites from Mallorca are not significantly different from each

other. Relative to Mallorcan individuals, individuals of the same size from Genoa and St Tropez tended to have a longer radula and smaller shell height. The Isle Rousse sample is the only sample that appears from the SNK test to have a group mean discrete from all other samples.

Principal component 4, which accounts for 1.3% of the variance in the data set, is dominated by a positive apex position element and a negative shell weight element (Table 4.3). Though there are significant differences between group means in this principal component (ANOVA $p<0.001$) there is little group specific differentiation (Fig 4.7).

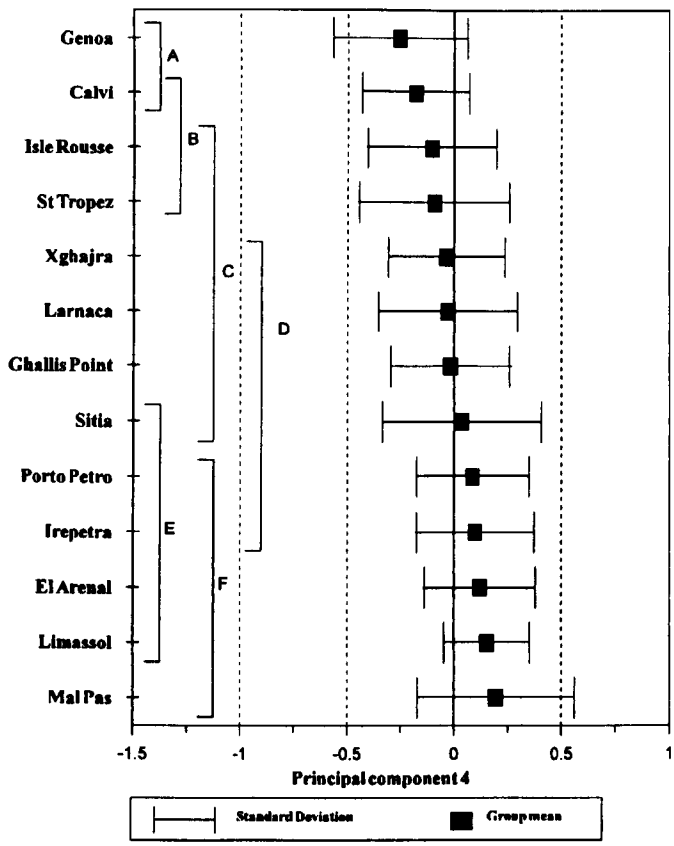


Figure 4.7 - Distribution of the group means of the thirteen samples of *P. caerulea* along principal component 4, with standard deviations shown. There are significant differences between groups ($F=21.39$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data has been $\log10(x+1)$ transformed and a correlation matrix used to standardise the data.

Bivariate plots of the sample group means (Fig 4.8 and 4.9) are used to look for country or regional differences that might be obscured by univariate plots. The only such cluster evident is in figure 4.9 of principal component 3 against principal component 4 where the Mallorcan samples are in a cluster in the top left of the graph. Relative to individuals of the

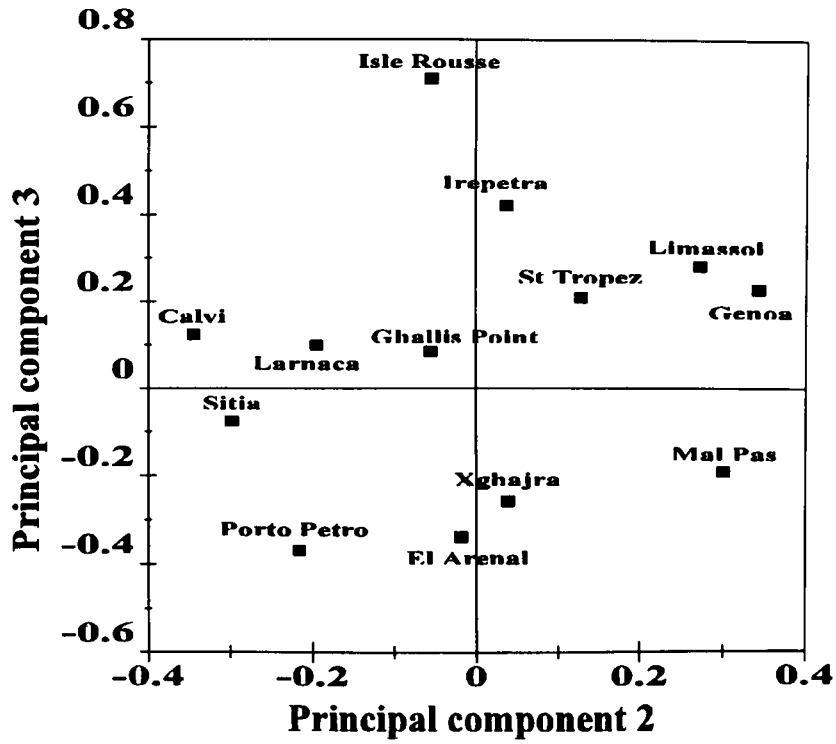


Figure 4.8 - Plot of principal component 2 against principal component 3 for the thirteen samples of *P. caerulea* from the Mediterranean. The data have been log10(x+1) transformed and a correlation matrix used to standardise the data. The data are separated by site.

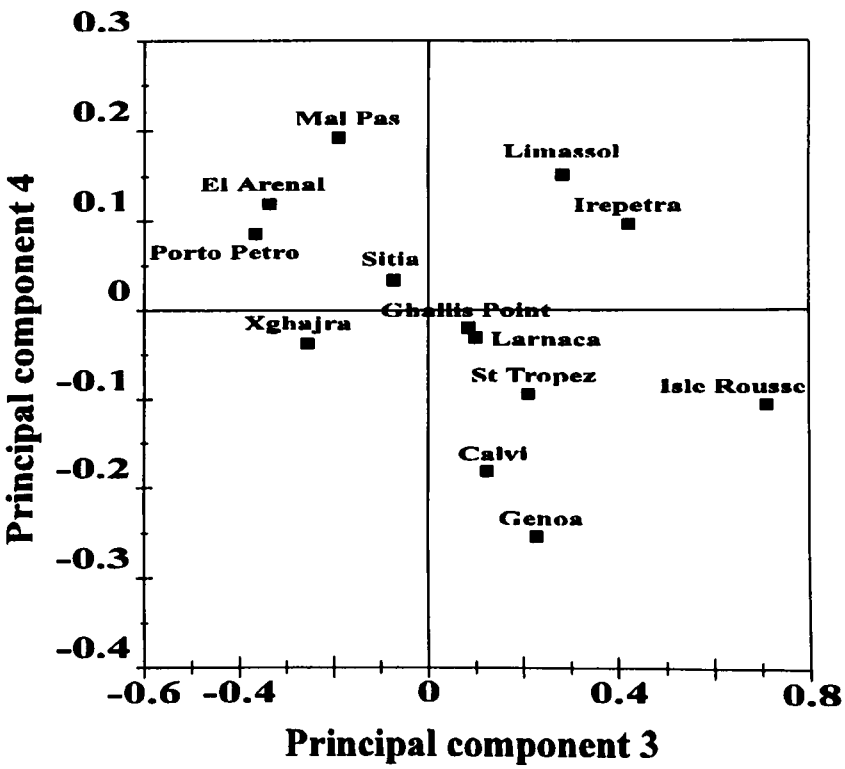


Figure 4.9 - Plot of principal component 2 against principal component 3 for the thirteen samples of *P. caerulea* from the Mediterranean. The data have been log10(x+1) transformed and a correlation matrix used to standardise the data. The data are separated by site.

same size from other samples Mallorcan individuals will have a shorter radula length and lower shell weight whilst having an apex closer to the posterior of the shell and a greater shell height.

Generally the degree of inter-group differentiation found by the principal component analysis is low. There is a large degree of overlap between the group distributions along all of the first four principal component axes, with no group obviously separate from all the others. There is no apparent regionally specific differentiation of sample sites.

4.34- Canonical Discriminant Analysis and Cross-validated Discriminant Function Analysis

Table 4.4 - Standardized canonical coefficients for *P. caerulea* samples from the thirteen sites throughout the Mediterranean differentiated by site. The data have been $\log_{10}(x+1)$ transformed and standardized.

Measured character	Canonical variate 1	Canonical variate 2	Canonical variate 3	Canonical variate 4	Canonical variate 5
Percentage of variance	46.3 %	21.6%	14.1%	7.0 %	5.4 %
Length	2.835	-0.299	-1.499	-2.966	0.561
Width (max)	0.371	-1.651	1.229	-0.394	1.729
Height (width)	-0.394	-2.892	1.689	-3.170	0.255
Height (length)	-0.229	4.251	-1.763	1.957	1.023
Apex	-1.007	0.690	1.296	0.684	0.615
Width (apex)	-1.319	1.349	1.464	2.376	-2.911
Radula	0.389	-0.994	-0.088	1.054	1.119
Cube root of weight	0.617	-0.112	-1.835	0.618	-2.095

The canonical variate analysis attempts to maximise the distances between the groups in multivariate space. This method should show any group specific differences more clearly. However, this is carried out without any information input as to the geographic position of the sites, so as not to 'force' any geographically related differences. Table 4.4 gives the percentage variance described by each of the first five canonical variates and the eigenvectors of each variate. Canonical variate 1 accounts for 46.3% of the variance, the dominant element in this canonical variate being shell length. Also contributing significantly are negative apex position and width across apex elements and to a lesser extent a positive weight element. The second canonical variate (21.6% of the variance) is dominated by shell height (along length) with significant negative contributions from maximum width and height (across

Table 4.5 - Canonical pairwise squared distances between groups for the thirteen *P. caerulea* samples from the Mediterranean differentiated by site. The data have been $\log_{10}(x+1)$ transformed and standardized.

Site	El Arenal	Mal Pas	Porto Petro	Calvi	Isle Rousse	St Tropez	Genoa	Ghallis Point	Xghajra	Irepetra	Sitia	Larnaca	Limassol
El Arenal	0												
Mal Pas	3.525	0											
Porto Petro	3.862	1.679	0										
Calvi	7.777	6.770	6.425	0									
Isle Rousse	8.863	11.247	12.725	4.161	0								
St Tropez	4.962	4.448	4.979	3.870	5.942	0							
Genoa	5.806	6.877	7.362	4.849	4.325	1.554	0						
Ghallis Point	9.756	4.036	3.266	11.000	16.408	7.907	10.883	0					
Xghajra	4.964	1.220	1.792	5.305	11.457	5.413	7.834	2.811	0				
Irepetra	6.431	3.704	5.654	3.809	4.156	5.461	6.555	6.387	3.440	0			
Sitia	4.243	9.305	9.448	6.651	4.719	10.200	8.840	16.256	9.102	5.350	0		
Larnaca	2.706	7.344	7.351	9.138	8.211	3.944	5.783	10.347	7.920	8.007	7.602	0	
Limassol	8.235	2.157	4.662	4.991	8.807	4.614	7.740	4.625	2.454	1.764	11.214	9.912	0

width) elements and a positive width across apex element. Canonical variate 3 has no single dominant element and is made up of all elements, bar radula length, to an equal order of magnitude. Canonical variate 4 is made up of a negative shell length and height (across width) element and a positive width across apex and height (along length) element.

Table 4.5 shows the pairwise squared distances between the groups in canonical space based on the canonical variate analysis of the shell parameters. The two closest groups are Xghajra and Mal Pas (distance=1.22) with the maximum distance between any two groups being 16.41 between Isle Rousse and Ghallis Point samples. Using a dendrogram to illustrate these distances (Fig 4.10), it is clear that the closest two groups are indeed Mal Pas and

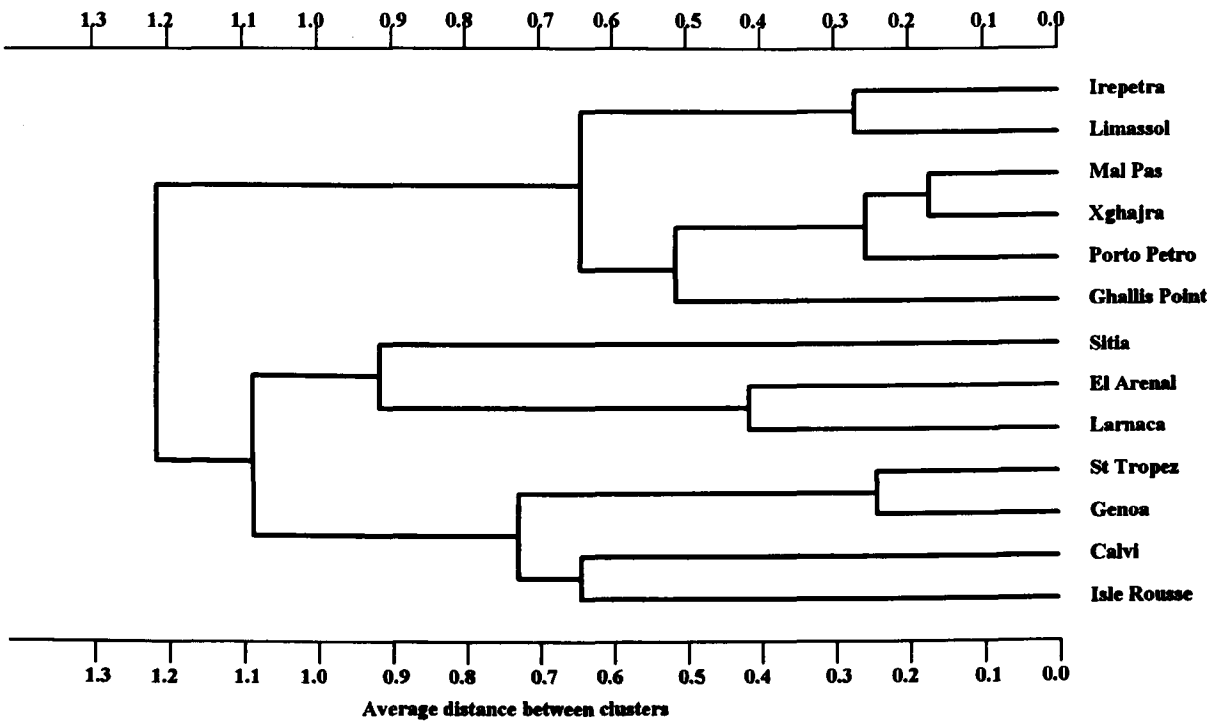


Figure 4.10 - Dendrogram based on a UPGMA cluster analysis of the pairwise squared canonical distances between the thirteen samples of *P.caerulea* from throughout the Mediterranean.

Xghajra, with St Tropez and Genoa being similarly related in shell shape and size. There is some relationship between the canonical variate analysis results and geographic location. The St Tropez sample is most similar to the Genoa sample in terms of shell size and shape and is from the same geographic region (see section 1). Similarly from the canonical variate analysis the two samples from Corsica group together. These four sample sites (St Tropez,

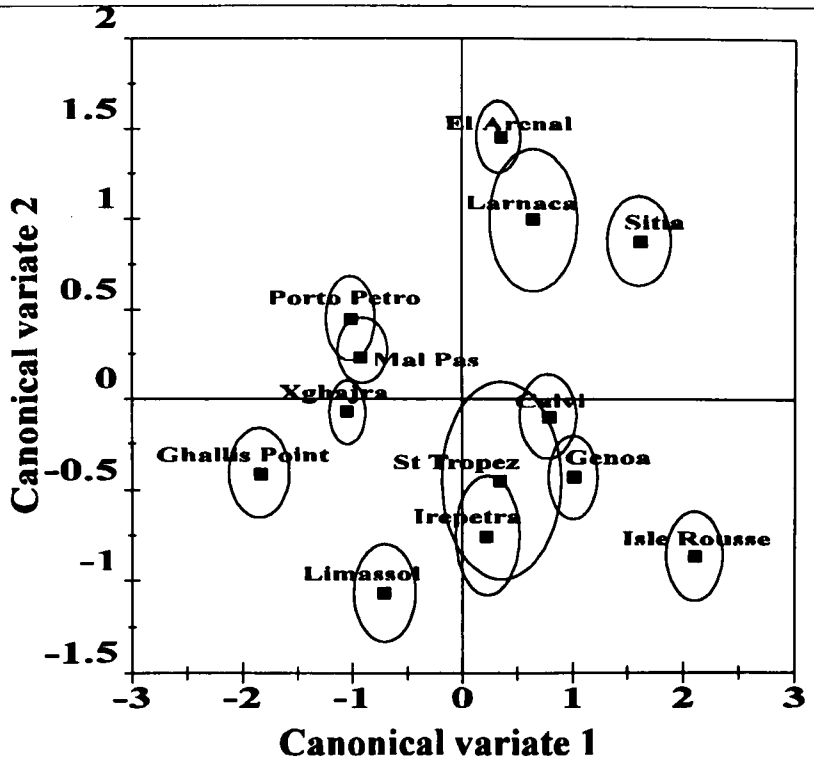


Figure 4.11 - Plot of canonical variate 1 against canonical variate 2 for the thirteen samples of *P. caerulea* from the Mediterranean. The data have been $\log_{10}(x+1)$ transformed and standardized. The data are separated by site, with group centroids and 95% confidence intervals of the mean shown.

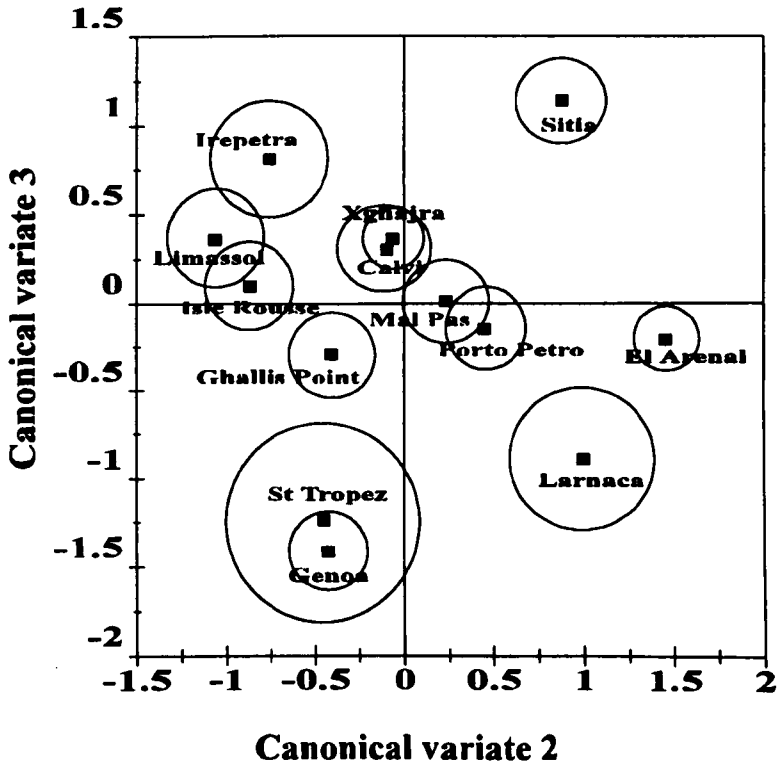


Figure 4.12 - Plot of canonical variate 2 against canonical variate 3 for the thirteen samples of *P. caerulea* from the Mediterranean. The data have been $\log_{10}(x+1)$ transformed and standardized. The data are separated by site, with group centroids and 95% confidence intervals of the mean shown.

Genoa, Calvi and Isle Rousse) form a cluster in the dendrogram and are quite close geographically. The Xghajra and Ghallis Point samples are also quite closely related in terms of shell size and shape. The canonical variate analysis simply maximises the distances between the sample groups. It follows that any grouping based on geographical location is due to similarity between the limpet shell shape at the sites concerned.

Table 4.6 - Group means of the first canonical variates for *P. caerulea* from the thirteen sites in the Mediterranean differentiated by site. The data have been $\log_{10}(x+1)$ transformed and standardized.

SITE	Canonical variate 1	Canonical variate 2	Canonical variate 3	Canonical variate 4	Canonical variate 5
El Arenal	0.356	1.455	-0.208	-0.012	0.226
Mal Pas	-0.922	0.232	0.015	-0.049	0.718
Porto Petro	-1.010	0.451	-0.148	-0.448	-0.379
Calvi	0.792	-0.100	0.303	-0.995	-0.283
Isle Rousse	2.103	-0.865	0.100	0.477	-0.037
St Tropez	0.349	-0.450	-1.239	-0.204	0.272
Genoa	1.015	-0.425	-1.414	-0.138	0.020
Ghallis Point	-1.835	-0.408	-0.294	0.744	-0.711
Xghajra	-1.048	-0.068	0.365	-0.221	-0.043
Irepetra	0.221	-0.754	0.812	0.656	0.188
Sitia	1.616	0.882	1.143	0.126	-0.318
Larnaca	0.647	0.999	-0.886	0.727	-0.207
Limassol	-0.708	-1.062	0.362	0.253	0.747

Bivariate plots were used to determine which canonical variates separate groups and therefore which shell characteristics differentiate between different groups (Fig 4.11, 4.12 & 4.13). These are based on the standardised canonical group means (Table 4.6) with 95% confidence intervals of the means shown. St Tropez and Genoa samples have similar group means along all of the first four canonical variates, with the group mean of each falling within the 95% confidence limits of the group mean of the other along each of the canonical variates. This indicates a similarity in most aspects of shell size and shape between these two samples. Calvi and Isle Rousse samples have similar group means along canonical variate 3. The canonical variate is a mixture of all the shell characters, and so does not give much useful information as to what are the similarities between these two samples. The Xghajra and Ghallis Point samples are similar along canonical variate 2; this being mainly a negative relationship between shell height (along length) and shell height (across width). Such a contrast

Table 4.5 - Results of a linear discriminant function analysis using cross-validation, showing the percentage of individuals from each sample correctly assigned to their original group (bold).

Site	El Arenal	Mal Pas	Porto Petro	Calvi	Isle Rousse	St Tropez	Genoa	Ghallis Point	Xghajra	Irepetra	Sitia	Larnaca	Limassol
El Arenal	**38.56**	6.54	10.46	3.27	0.00	3.92	3.27	0.65	3.92	1.96	15.69	11.76	0.00
Mal Pas	4.55	**27.27**	18.18	0.91	0.00	4.55	2.73	6.36	10.91	5.45	0.91	3.64	14.55
Porto Petro	11.82	1.82	**45.45**	2.73	0.00	0.91	0.00	11.82	14.55	5.45	1.82	2.73	0.91
Calvi	0.00	0.00	6.19	**52.58**	7.22	3.09	7.22	1.03	4.12	7.22	6.19	0.00	5.15
Isle Rousse	0.00	0.00	1.01	15.15	**51.52**	3.03	4.04	3.03	0.00	8.08	11.11	3.03	0.00
St Tropez	0.00	0.00	15.00	5.00	5.00	**20.00**	25.00	5.00	0.00	0.00	5.00	15.00	5.00
Genoa	8.55	2.56	2.56	3.42	5.13	14.53	**56.41**	0.00	1.71	3.42	0.00	085	0.85
Ghallis Point	1.00	4.00	13.00	0.00	1.00	2.00	0.00	**61.00**	4.00	2.00	0.00	4.00	8.00
Xghajra	0.50	11.56	11.56	2.01	0.00	2.51	1.51	8.54	**44.72**	5.03	1.01	3.52	7.54
Irepetra	0.00	5.45	1.82	14.55	5.45	1.82	7.27	1.82	3.64	**32.73**	10.91	3.64	10.91
Sitia	7.07	0.00	0.00	3.03	6.06	1.01	0.00	1.01	0.00	8.08	**46.49**	4.04	1.01
Larnaca	24.32	8.11	0.00	2.70	2.70	5.41	2.70	2.70	2.70	2.70	0.00	**45.95**	0.00
Limassol	0.00	8.75	2.50	6.25	2.50	5.00	0.00	11.25	3.75	15.00	0.00	0.00	**45.00**
% allocated to group	8.23	6.66	10.82	7.92	6.11	4.23	7.68	9.09	10.89	6.66	9.48	5.17	7.05
% of data set	11.99	8.62	8.62	7.60	7.76	1.57	9.17	7.84	15.60	4.31	7.76	2.90	6.27

of the two height characters is descriptive of the curvature in the basal plane of the limpet shells and thus the two samples have a similar degree of curvature in the basal plane. The differences between the groups along the canonical variates could be described individually, but as this study is concentrating on geographical relationships this would serve little purpose. It is sufficient to note that there is no clear geographical pattern to the data set. There is certainly no cline of shape differences throughout the Mediterranean. Also there is a much

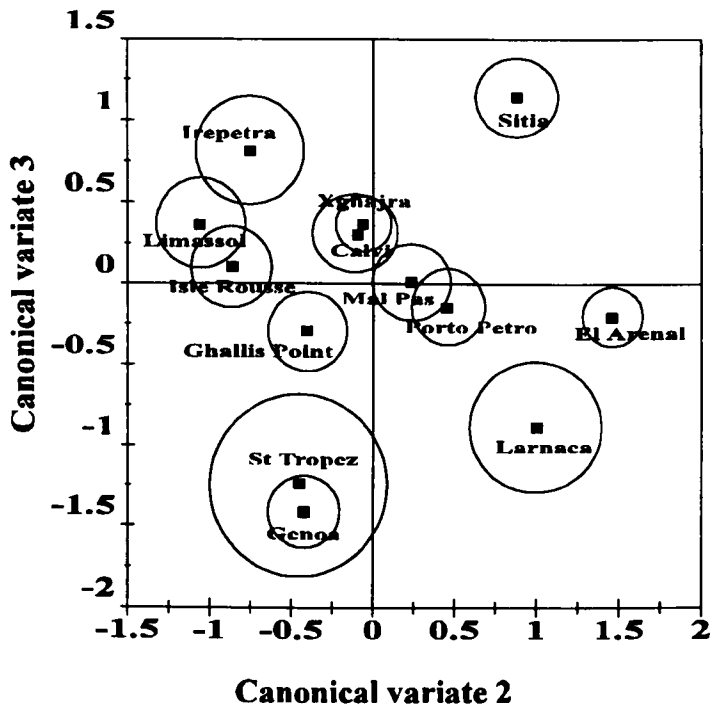


Figure 4.12 - Plot of canonical variate 2 against canonical variate 3 for the thirteen samples of *P. caerulea* from the Mediterranean. The data have been $\log_{10}(x+1)$ transformed and standardized. The data are separated by site, with group centroids and 95% confidence intervals of the mean shown.

lower level of differentiation between groups from vastly disparate geographical locations within the Mediterranean, than there is between samples from different heights on the same shore (see chapter 3). The low differentiation is shown clearly by the results of the linear discriminant function analysis (Table 4.7). The maximum percentage of limpets within a sample correctly classified to a site is 68.69% (compared with 92.77 for classification into the infralittoral zone in chapter 3) and the majority of samples have a correct assignment percentage below 50%. The lowest percentage assigned correctly is 20% (within the St Tropez sample). This particularly low number is partially due to the closeness of this sample and the Genoa sample in canonical space, with 25% of the St Tropez sample being assigned to Genoa. Also the small sample size from St Tropez will affect the results. There is no geo-

graphically orientated pattern to the groups into which incorrectly assigned individuals are placed, if geographical location heavily influenced shell shape some correlation would be expected.

There are clearly no apparent geographically dictated patterns in shell size and shape. It also appears that the degree of differentiation between samples is very low, with high levels of overlap in the distributions of samples along both principal component and canonical variate axes and low levels of correct assignment in the linear discriminant function analysis.

4.3.5 - Electrophoretic analysis

The allele frequencies of the loci used in this study are given in Table 4.8, of these loci six were shown to be consistently polymorphic. At the *G3pdh* locus allele C only occurred in the samples from Calvi and Isle Rousse, and the Isle Rousse sample was the only one to show allele E at the *Pgdh* locus. The Limassol sample showed alleles unique to the sample at two loci (allele D at *Np* locus and allele D at *Mpi* locus). *Mpi* was fixed for two samples, Ghallis Point and Xghajra. The *Mdh-1*, *Mdh-2*, *Ald-1*, *Idh-1*, *Idh-2*, *Me-2* and *Got* loci are fixed for a single allele in all thirteen samples. The mean number of alleles per locus (Table 4.9) ranged from 1.5 to 2.0 whilst the percentage of polymorphic loci ranged from 30.8 to 46.2. Using Nei's unbiased estimate of expected heterozygosity it is clear that all samples showed a heterozygote deficitency when averaged over all loci. Looking at the heterozygote deficiency at the locus level (Table 4.10), no sample showed a significant heterozygote excess at any of the six polymorphic loci. A heterozygote excess would be indicated by a positive D value (negative disequilibrium coefficient) and a significant χ^2 result. Where rare alleles were present that could bias the results they were pooled, which is indicated in the right hand column of the table. Samples from Irepetra, Sitia, Genoa, Mal Pas, Porto Petro, Ghallis Point and Limassol all had three or more loci that show a significant ($p < 0.01$) heterozygote deficiency. In no sample were all loci heterozygote deficient ($p < 0.01$). The greatest deficiencies were shown by Irepetra, Sitia and Limassol.

Nei's (1972) unbiased genetic identity was used to show the relatedness of samples (Table 4.11). The level of genetic identity ranged between 0.999 (between St Tropez and Genoa and between Mal Pas and Porto Petro) and 0.831 (between Ghallis Point and El Arenal). A UPGMA dendrogram based on Nei's unbiased genetic identity (Fig 4.14) shows the samples

from the same country to be the closest related. Samples from St Tropez and Genoa were both most closely related to those from Malta. Samples from Corsica were most closely related to those from Mallorca. The most closely related samples of limpets are the two from the Eastern Mediterranean basin: Crete and Cyprus. Though the samples from the Eastern Mediterranean basin form a cluster in the dendrogram (Fig 4.14), there is no obvious geographically dependent relationship between samples from different countries.

Analysis of heterogeneity of allele frequencies (Table 4.12) looks for significant differences in allele frequencies between samples from the same country. At the 1% level Italy, Malta, Crete and Cyprus show no significant differences in allele frequencies between samples. Variation in allele frequencies at the PGM-1 locus in samples from Mallorca and Cor-

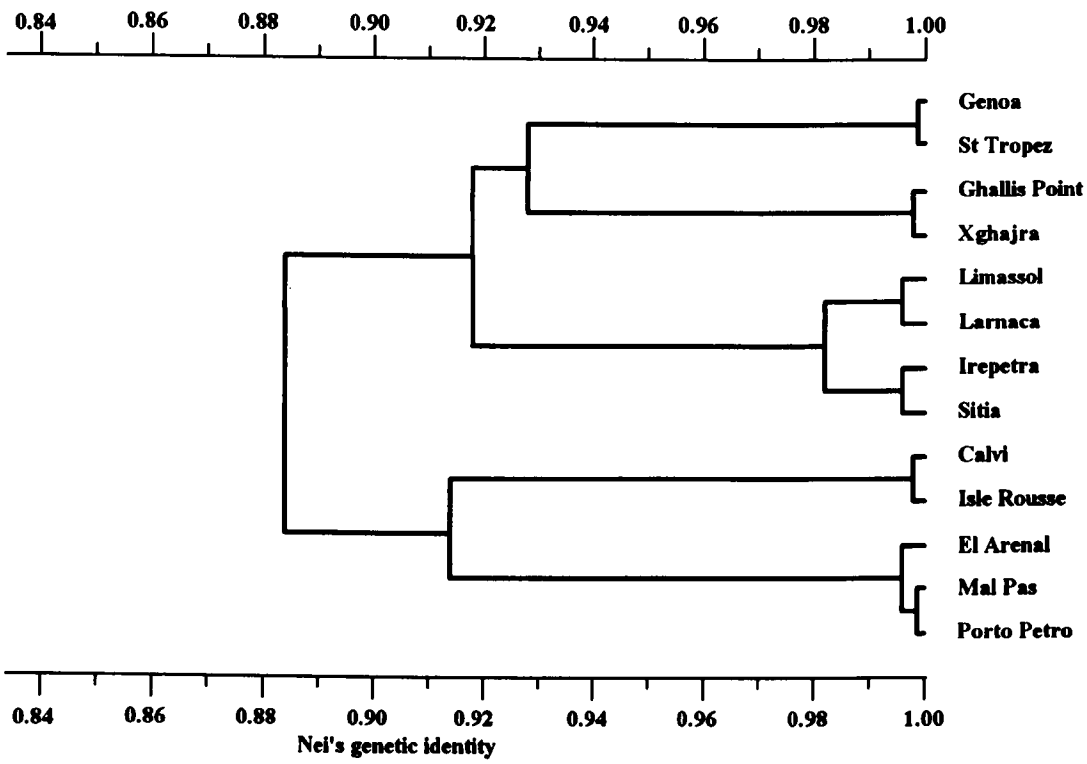


Figure 4.14 - Dendrogram based on a UPGMA cluster analysis of Nei's (1978) genetic identity between the thirteen samples of *P. caerulea*.

sica indicate an overall significant degree of heterogeneity in the samples from these two countries.

Figure 4.8 - Allele frequencies and sample size (N) at thirteen loci for the thirteen samples of *P.caerulea* from throughout the Mediterranean.

SITE	1	2	3	4	5	6	7	8	9	10	11	12	13
Locus													
Pgi-1													
(N)	**113**	**112**	**112**	**97**	**85**	**20**	**70**	**78**	**76**	**55**	**77**	**20**	**79**
A	0.000	0.027	0.000	0.113	0.159	0.150	0.164	0.173	0.132	0.364	0.214	0.200	0.316
B	0.836	0.688	0.763	0.876	0.818	0.750	0.829	0.827	0.868	0.600	0.740	0.775	0.658
C	0.164	0.286	0.237	0.000	0.006	0.000	0.000	0.000	0.000	0.036	0.045	0.025	0.025
D	0.000	0.000	0.000	0.010	0.018	0.100	0.007	0.000	0.000	0.000	0.000	0.000	0.000
Pgm-1													
(N)	**108**	**95**	**100**	**93**	**93**	**20**	**73**	**75**	**65**	**50**	**58**	**16**	**66**
A	0.000	0.000	0.000	0.054	0.043	0.500	0.527	0.487	0.377	0.280	0.362	0.500	0.462
B	0.028	0.232	0.150	0.758	0.796	0.350	0.178	0.207	0.146	0.180	0.216	0.344	0.242
C	0.213	0.116	0.140	0.124	0.000	0.000	0.027	0.027	0.031	0.220	0.267	0.000	0.106
D	0.759	0.653	0.710	0.065	0.161	0.150	0.267	0.280	0.446	0.320	0.155	0.156	0.189
G3pdh													
(N)	**111**	**106**	**110**	**90**	**100**	**20**	**79**	**61**	**74**	**45**	**82**	**20**	**54**
A	0.005	0.028	0.041	0.156	0.035	0.875	0.930	0.959	0.851	0.244	0.146	0.450	0.667
B	0.167	0.208	0.182	0.800	0.880	0.125	0.070	0.041	0.149	0.756	0.854	0.550	0.333
C	0.829	0.764	0.777	0.044	0.085	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pgdh													
(N)	**107**	**87**	**97**	**70**	**62**	**20**	**70**	**55**	**55**	**46**	**74**	**20**	**80**
A	0.687	0.615	0.655	0.900	0.855	0.875	0.893	0.836	0.791	0.565	0.608	0.625	0.619
B	0.299	0.379	0.294	0.086	0.129	0.025	0.086	0.164	0.209	0.272	0.250	0.300	0.231
C	0.014	0.006	0.052	0.014	0.008	0.100	0.021	0.000	0.000	0.163	0.142	0.075	0.150
D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
E	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Np													
(N)	**99**	**90**	**94**	**97**	**99**	**20**	**69**	**80**	**80**	**55**	**82**	**20**	**79**
A	0.086	0.078	0.032	0.088	0.045	0.100	0.043	0.050	0.094	0.064	0.091	0.025	0.082
B	0.914	0.922	0.968	0.912	0.955	0.900	0.957	0.950	0.906	0.936	0.909	0.975	0.905
C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.013
Mpi-1													
(N)	**94**	**94**	**99**	**80**	**78**	**20**	**82**	**50**	**50**	**49**	**86**	**20**	**80**
A	0.090	0.149	0.202	0.081	0.109	0.050	0.049	1.000	1.000	0.337	0.233	0.325	0.225
B	0.910	0.851	0.798	0.844	0.846	0.775	0.793	0.000	0.000	0.000	0.035	0.000	0.000
C	0.000	0.000	0.000	0.075	0.045	0.175	0.159	0.000	0.000	0.663	0.733	0.675	0.769
D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
Mdh-1													
(N)	**97**	**89**	**98**	**97**	**99**	**20**	**82**	**78**	**80**	**55**	**87**	**20**	**80**
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Mdh-2													
(N)	**97**	**89**	**98**	**97**	**99**	**20**	**82**	**78**	**80**	**55**	**87**	**20**	**80**
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Ald-1													
(N)	**97**	**89**	**98**	**97**	**99**	**20**	**82**	**78**	**80**	**55**	**87**	**20**	**80**
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Idh-1													
(N)	**97**	**89**	**98**	**97**	**99**	**20**	**82**	**78**	**80**	**55**	**87**	**20**	**80**
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Figure 4.8 (continued) - Allele frequencies and sample size (N) at thirteen loci for the thirteen samples of *P.caerulea* from throughout the Mediterranean.

SITE	1	2	3	4	5	6	7	8	9	10	11	12	13
Locus													
Idh-2													
(N)	**97**	**89**	**98**	**97**	**99**	**20**	**82**	**78**	**80**	**55**	**87**	**20**	**80**
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Me-2													
(N)	**97**	**89**	**98**	**97**	**99**	**20**	**82**	**78**	**80**	**55**	**87**	**20**	**80**
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Got													
(N)	**97**	**89**	**98**	**97**	**99**	**20**	**82**	**78**	**80**	**55**	**87**	**20**	**80**
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Key to Sites

1 - El Arenal, Mallorca
2 - Mal Pas, Mallorca
3 - Porto Petro, Mallorca
4 - Calvi, Corsica
5 - Isle Rousse, Corsica
6 - St Tropez, France
7 - Genoa, Italy
8 - Ghallis Point, Malta
9 - Xghajra, Malta
10 - Irepetra, Crete
11 - Sitia, Crete
12 - Larnaca, Cyprus
13 - Limassol, Cyprus

Table 4.9 - Summary of the genetic variability in the thirteen *P.caerulea* samples from the Mediterranean. Shown are the mean sample size per locus; the mean number of alleles per locus; percentage of polymorphic loci (Commonest allele does not exceed 95%) and the mean observed and expected (Nei's unbiased estimate, 1978) heterozygosity proportions.

Population	Mean Sample size per locus	Mean No. of alleles per locus	Percentage of loci polymorphic	Mean Heteozygosity Observed	Mean Heteozygosity H-W expected
El Arenal Mallorca	100.8	1.7	46.2	0.104	0.131
S.E.	±1.8	±0.2		±0.037	±0.046
Mal Pas Mallorca	92.8	1.8	46.2	0.130	0.170
S.E.	±2.1	±0.3		±0.046	±0.059
Porto Petro Mallorca	99.8	1.7	38.5	0.118	0.158
S.E.	±1.4	±0.2		±0.042	±0.056
Calvi Corsica	92.8	1.9	46.2	0.099	0.122
S.E.	±2.3	±0.3		±0.033	±0.042
Isle Rousse Corsica	93.1	2.0	38.5	0.088	0.114
S.E.	±3.2	±0.3		±0.030	±0.039
St Tropez France	20.0	1.8	46.2	0.131	0.158
S.E.	±0.0	±0.3		±0.056	±0.057
Genoa Italy	40.0	1.8	38.5	0.122	0.128
S.E.	±1.5	±0.3		±0.064	±0.053
Ghallis Point Malta	72.7	1.5	30.8	0.075	0.107
S.E.	±2.8	±0.2		±0.037	±0.053
Xghajra Malta	73.8	1.5	38.5	0.098	0.125
S.E.	±2.9	±0.2		±0.042	±0.055
Irepetra Crete	52.7	1.8	46.2	0.087	0.215
S.E.	±1.1	±0.3		±0.035	±0.077
Sitia, Crete	82.2	1.8	46.2	0.093	0.194
S.E.	±2.3	±0.3		±0.038	±0.071
Larnaca Cyprus	19.7	1.7	38.5	0.167	0.195
S.E.	±0.3	±0.2		±0.064	±0.071
Limassol Cyprus	76.8	1.9	46.2	0.115	0.206
S.E.	±2.2	±0.3		±0.049	±0.071

Table 4.10 - Summary statistic of heterozygosity levels for the polymorphic loci in the thirteen *P. caerulea* samples. Expected heterozygosite is calculated using Nei's unbiased estimate of heterozygosity. The loci fixation index (F) and disequilibrium coefficient (D) are given and the results of a $\chi 2$ test for significant departure from expected heterozygosity level.

Locus	Observed hetero-zygotes	Expected hetero-zygotes	Fixation index (F)	D	$\chi 2$	D.F.	*P*	Pooled
El Arenal								
Pgi-1	25	31.08	0.192	-0.196	4.43	1	*	
Pgm-1	32	40.95	0.215	-0.219	9.49	1	**	√
G3pdh	25	31.81	0.210	-0.214	6.62	1	*	√
Pgdh	39	47.14	0.169	-0.173	2.66	1	NS	√
Np	11	15.62	0.292	-0.296	9.16	1	**	
Mpi-1	13	15.55	0.159	-0.164	2.67	1	NS	
Mal Pas								
Pgi-1	42	50.64	0.157	-0.161	3.37	1	NS	√
Pgm-1	36	48.42	0.253	-0.257	19.12	1	***	√
G3pdh	27	39.64	0.316	-0.319	15.15	1	***	√
Pgdh	35	41.82	0.158	-0.163	2.13	1	NS	√
Np	10	12.98	0.225	-0.236	5.09	1	NS	
Mpi-1	16	23.96	0.329	-00.332	10.71	1	**	
Porto Petro								
Pgi-1	31	40.64	0.234	-0.237	6.40	1	*	
Pgm-1	33	45.61	0.273	-0.276	27.09	1	***	√
G3pdh	26	39.90	0.345	-0.348	28.39	1	***	√
Pgdh	39	47.04	0.167	-0.171	2.54	1	NS	√
Np	6	5.84	-0.033	0.027	0.09	1	NS	
Mpi-1	22	32.08	0.311	-0.314	9.99	1	**	
Calvi								
Pgi-1	20	21.37	0.059	-0.064	0.29	1	NS	√
Pgm-1	30	37.68	0.200	-0.204	2.26	1	NS	√
G3pdh	24	30.21	0.201	-0.206	2.71	1	NS	√
Pgdh	10	12.86	0.217	-0.223	3.37	1	NS	√
Np	15	15.59	0.033	-0.038	0.15	1	NS	
Mpi-1	16	22.21	0.275	-0.286	7.13	1	NS	√
Isle Rousse								
Pgi-1	20	26.15	0.231	-0.235	5.68	1	*	√
Pgm-1	22	31.70	0.302	-0.306	11.13	1	**	√
G3pdh	16	21.82	0.263	-0.267	6.30	1	*	√
Pgdh	14	15.78	0.106	-0.113	0.62	1	NS	√
Np	7	8.64	0.185	-0.189	3.97	1	*	
Mpi-1	17	21.21	0.193	-0.198	3.82	1	NS	√
St Tropez								
Pgi-1	6	8.31	0.259	-0.278	1.06	1	NS	√
Pgm-1	14	12.41	-0.157	0.128	0.61	1	NS	√
G3pdh	3	4.49	0.314	-0.331	2.69	1	NS	
Pgdh	5	4.59	-0.117	0.089	0.32	1	NS	√
Np	2	3.69	0.444	-0.458	5.47	1	*	
Mpi-1	4	7.51	0.454	-0.468	7.45	1	**	√

Table 4.10 (continued) - Summary statistic of heterozygosity levels for the polymorphic loci in the thirteen *P. caerulea* samples. Expected heterozygosite is calculated using Nei's unbiased estimate of heterozygosity. The loci fixation index (F) and disequilibrium coefficient (D) are given and the results of a $\chi 2$ test for significant departure from expected heterozygosity level.

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	D	$\chi 2$	D.F.	*P*	Pooled
Genoa								
Pgi-1	16	20.19	0.202	-0.208	2.94	1	NS	√
Pgm-1	61	45.43	-0.352	0.343	22.85	1	***	√
G3pdh	7	10.30	0.316	-0.320	8.87	1	**	
Pgdh	11	13.75	0.194	-0.200	2.54	1	NS	√
Np	4	5.78	0.303	-0.308	7.80	1	**	
Mpi-1	18	28.39	0.362	-0.366	14.13	1	***	√
Ghallis Point								
Pgi-1	15	22.47	0.328	-0.332	8.91	1	**	
Pgm-1	33	48.42	0.314	-0.319	6.14	1	*	√
G3pdh	3	4.84	0.374	-0.379	10.89	1	**	
Pgdh	12	15.19	0.203	-0.210	2.55	1	NS	
Np	6	7.65	0.211	-0.215	4.22	1	*	
Xghajra								
Pgi-1	14	17.48	0.194	-0.199	3.16	1	NS	
Pgm-1	31	41.70	0.251	-0.257	2.56	1	NS	√
G3pdh	20	18.86	-0.068	0.061	0.28	1	NS	
Pgdh	13	18.36	0.285	-.292	4.87	1	*	
Np	9	13.68	0.338	-0.344	9.97	1	**	
Irepetra								
Pgi-1	10	28.11	0.641	-0.644	29.37	3	***	√
Pgm-1	19	37.29	0.485	-0.491	6.77	1	**	√
G3pdh	6	16.81	0.639	-0.643	19.35	1	***	
Pgdh	13	26.98	0.513	-0.518	14.89	1	***	√
Np	5	6.62	0.237	-0.244	3.79	1	NS	
Mpi-1	3	22.11	0.863	-0.864	37.56	1	***	
Sitia								
Pgi-1	12	31.31	0.614	-0.617	34.72	1	***	√
Pgm-1	25	42.53	0.407	-0.412	6.60	1	*	√
G3pdh	10	20.61	0.512	-0.515	22.57	1	***	
Pgdh	22	40.80	0.457	-0.461	10.87	1	**	√
Np	9	13.71	0.340	-0.344	10.32	1	**	
Mpi-1	8	35.30	0.772	-0.772	51.12	1	***	√
Larnaca								
Pgi-1	6	7.36	0.164	-0.185	2.00	1	NS	√
Pgm-1	10	10.03	-0.029	-0.003	0.76	1	NS	√
G3pdh	10	10.15	-0.010	-0.015	0.01	1	NS	
Pgdh	9	10.54	0.124	-0.146	0.09	1	NS	√
Np	1	1.00	-0.026	0.000	0.00	1	NS	
Mpi-1	5	9.00	0.430	-0.444	4.22	1	*	

Table 4.10 (continued) - Summary statistic of heterozygosity levels for the polymorphic loci in the thirteen *P. caerulea* samples. Expected heterozygosite is calculated using Nei's unbiased estimate of heterozygosity. The loci fixation index (F) and disequilibrium coefficient (D) are given and the results of a $\chi 2$ test for significant departure from expected heterozygosity level.

Locus	Observed hetero-zygotes	Expected hetero-zygotes	Fixation index (F)	D	$\chi 2$	D.F.	*P*	Pooled
Limassol								
Pgi-1	6	37.05	0.837	-0.838	63.30	1	***	√
Pgm-1	37	45.26	0.176	-0.182	0.23	1	NS	√
G3pdh	12	24.22	0.500	-0.505	14.08	1	***	
Pgdh	29	43.57	0.336	-0.334	4.54	1	*	√
Np	13	13.83	0.054	-0.060	0.20	1	NS	√
Mpi-1	9	28.85	0.686	-0.688	38.47	1	***	√

Table 4.11 - Nei's unbiased genetic identity over thirteen loci between the thirteen *P. caerulea* samples from the Mediterranean.

Site	El Arenal	Mal Pas	Porto Petro	Calvi	Isle Rousse	St Tropez	Genoa	Ghallis Point	Xghajra	Irepetra	Sitia	Larnaca	Limassol
El Arenal	0												
Mal Pas	0.995	0											
Porto Petro	0.998	0.999	0										
Calvi	0.904	0.920	0.914	0									
Isle Rousse	0.905	0.924	0.918	0.998	0								
St Tropez	0.893	0.902	0.902	0.940	0.925	0							
Genoa	0.898	0.903	0.905	0.923	0.908	0.999	0						
Ghallis Point	0.831	0.844	0.854	0.855	0.844	0.931	0.932	0					
Xghajra	0.850	0.862	0.872	0.863	0.854	0.924	0.928	0.998	0				
Irepetra	0.865	0.881	0.883	0.915	0.914	0.904	0.898	0.904	0.916	0			
Sitia	0.858	0.872	0.873	0.925	0.921	0.901	0.892	0.883	0.895	0.997	0		
Larnaca	0.857	0.875	0.877	0.921	0.916	0.938	0.931	0.935	0.939	0.990	0.989	0	
Limassol	0.846	0.862	0.864	0.894	0.884	0.945	0.940	0.934	0.934	0.980	0.973	0.995	0

Table 4.12 - Summary of Chi-squared statistics for the analysis of heterogeneity of allele frequencies over six polymorphic loci between the samples from the same country. Shown are Number of alleles, Chi-squared value, degrees of freedom (D.F.) and probability level (p), where N.S. = not significant.

Mallorca

Locus	No. of alleles	χ^2	D.F.	*p*
Pgi-1	3	22.74	4	*
Pgm-1	3	40.82	4	***
G3pdh	3	7.82	4	NS
Pgdh	3	12.78	4	NS
Np	2	5.26	2	NS
Mpi-1	2	9.52	2	NS
(Totals)		98.95	20	***

Corsica

Locus	No. of alleles	χ^2	D.F.	*p*
Pgi-1	4	3.25	3	NS
Pgm-1	4	31.11	3	***
G3pdh	3	18.04	2	**
Pgdh	4	2.67	3	NS
Np	2	2.81	1	NS
Mpi-1	3	1.83	2	NS
(Totals)		59.71	14	***

France & Italy

Locus	No. of alleles	χ^2	D.F.	*p*
Pgi-1	3	9.93	2	NS
Pgm-1	4	7.24	3	NS
G3pdh	2	1.32	1	NS
Pgdh	3	6.54	2	NS
Np	2	1.87	1	NS
Mpi-1	3	0.67	2	NS
(Totals)		26.96	11	*

Table 4.12 (continued) - Summary of Chi-squared statistics for the analysis of heterogeneity of allele frequencies over six polymorphic loci between the samples from the same country. Shown are Number of alleles, Chi-squared value, degrees of freedom (D.F.) and probability level (p), where N.S. = not significant.

Malta

Locus	No. of alleles	χ^2	D.F.	*p*
Pgi-1	2	1.03	1	NS
Pgm-1	4	8.78	3	*
G3pdh	2	8.61	1	*
Pgdh	2	0.75	1	NS
Np	2	2.3	1	NS
(Totals)		21.46	7	*

Crete

Locus	No. of alleles	χ^2	D.F.	*p*
Pgi-1	3	7.16	2	*
Pgm-1	4	8.25	3	*
G3pdh	2	3.77	1	NS
Pgdh	3	0.45	2	NS
Np	2	0.69	1	NS
Mpi-1	3	6.35	2	*
(Totals)		26.66	11	NS

Cyprus

Locus	No. of alleles	χ^2	D.F.	*p*
Pgi-1	3	2.12	2	NS
Pgm-1	4	4.64	3	NS
G3pdh	2	5.75	1	*
Pgdh	3	1.95	2	NS
Np	3	2.16	2	NS
Mpi-1	3	1.93	2	NS
(Totals)		18.54	12	NS

4.3.6 - Further use of Principal component analysis.

The range of results for Nei's unbiased genetic identity is consistent with the suggestion that all samples are conspecific. With this in mind, and due to the low between-group differentiation in shell shape, one can re-examine the principal component analysis results looking at the data set as a whole. As principal component analysis treats the samples as a single data set the principal components generated will describe the variance in the data set as a whole. With the samples having been collected from a variety of mesolittoral microhabitats at each site and the sites covering the majority of the geographic range of *P. caerulea*, it is reasonable to assume that the majority of possible shape variations exhibited by the species will be found within the data set as a whole. The principal components generated by the analysis can therefore be used to describe the nature of the range of shape and size variation that *P. caerulea* exhibits as a species. The vast majority of the variance is due to size, with principal component 1 approximating to an isometric size vector (see earlier in results section) and describing 88.5% of the variance. Thus, as one would expect, the most variable shell character in *P. caerulea* is size. 4.4% of the variation (principal component 2) is a negative correlation between radula length (and to a lesser extend shell height) and shell basal area. A further 4.0% (principal component 3) of the variation is a negative correlation between radula length and shell height and 1.3% (principal component 4) is a negative correlation between shell weight and the distance between the apex and the anterior of the shell.

4.4 - Discussion

4.4.1 - Heterozygote deficiencies and heterogeneity of allele frequencies

The first point to be addressed with respect to the genetic results is the heterozygote deficiencies displayed by all samples but particularly those from Irepetra, Sitia and Limassol. Several reasons can be forwarded to explain such deficits.

The presence of null alleles has been proposed as one explanation for a heterozygote deficit (Skibinski *et al.* 1983). It has been demonstrated that a failure to score null alleles can result in a heterozygote deficit where otherwise an excess was shown to exist (Katoh and Foltz 1988). Null alleles are normally evidenced by occasional blanks on gels, as no blanks were found in this study the presence of null alleles is unlikely to be the reason for the het-

erozygote deficiencies found. However, such blanks can be rare and are easily missed when scoring a gel, so this reason cannot be dismissed out of hand.

Negative heterosis is another explanation given for heterozygote deficiencies (Volckaert and Zouros 1989; Beaumont and Zouros 1990) but the lack of any cohort or growth rate information makes it impossible to assess whether this contributes to the heterozygote deficiencies found in this study.

Another common reason given for heterozygote deficiencies is inbreeding. However in a species that is a protandrous hermaphrodite (thus precluding self-fertilisation), with external fertilisation and a planktonic larvae, inbreeding is intuitively unlikely.

Aneuploidy - the lose of a chromosome or segment of a chromosome - has been given as an explanation of heterzygote deficiencies in the oyster *Crassostrea gigas* (Thiriot-Quievreux *et al.* 1988),as has molecular imprinting - differential expression of a gene depending upon whether it is maternally or paternally derived (Chakraborty 1989).

The most likely explanation for the heterozygote deficiencies found in this study is the Wahlund effect (Wahlund 1928) see (Koehn and Mitton 1972; Tracey *et al.* 1975; Beaumont 1982; Johnson and Black 1982, 1984b). This effect occurs if two or more populations are sampled simultaneously where the alleles frequencies at the loci studied are different for the different populations. The resultant deficit of heterozygotes will be equal to the variance between source populations summed across all loci. These different populations may be different genetic stocks, but this is not necessarily the case. Johnson & Black (1984b) suggested that the Wahlund effect occurred in limpets where the limpets had a bimodal distribution on the shore, with a high and a low shore group. Mixing of different aged cohorts can also give a Wahlund effect (Tracey *et al.* 1975; Milkman and Koehn 1977; Crisp 1978). In the case of *P. caerulea* there are a number of factors that could contribute to a Wahlund effect; firstly the fact that *P. caerulea* has a planktonic larva. The length of larval life stage is not known, but is probably in the order of 10 days, this being the duration of the larval stage of *P. vulgata* in the laboratory (Dodd 1957). The current patterns in the Mediterranean, particularly around the islands, provide ample opportunity for mixing of water bodies and therefore mixing of larvae from different sources within the time period for which the larvae are in the water column. This could lead to a single settlement of larvae at a site that originated from a

number of different sources. Secondly variations in the current patterns in successive years, between the spawning and settlement periods, could lead to the larval supply in successive years coming from different sources. The bimodal distribution of *P. caerulea* (see chapter 3) where selection pressures in the two zones are probably very different could contribute to a Wahlund effect (Johnson and Black 1984b). Finally the fact that it is not easy to age patellids means that one cannot tell whether a number of cohorts are being sampled and thus mixing of genetically different age classes could contribute to a Wahlund effect. Differences in selection from year to year were proposed as a mechanism for maintaining such a 'temporal' Wahlund effect in the clam *Ruditapes decussatus* (Borsa *et al.* 1991). Another factor that could contribute to a Wahlund effect is the spawning cycle of *P. caerulea*. Spawning of *P. caerulea* at Naples extends from the beginning of November to May (Bacci 1947), spawning events being triggered by rough seas. With this protracted spawning season and the stochastic nature of spawning events, as well as seasonal variation in current patterns, it is likely that the source of larval supply to any location will vary during the year. Thus it is probable that the supply of larvae during a single year will come from genetically different sources, contributing to a Wahlund effect.

Another explanation offered to account for heterozygote deficiencies is selection against heterozygotes. This has been proposed as a mechanism for generating the heterozygote deficiencies found in mussels. Based on laboratory pair mating experiments of the mussel *Mytilus edulis*, Mallet *et al.* (1985) suggested that such selection occurred in the larval stage, whilst others suggest that such selection occurs after settlement and metamorphosis of the mussel (Beaumont 1991). It is generally agreed that if selection against heterozygotes occurs it is in the larval stage or early settlement (Gosling and Wilkens 1985; Fairbrother and Beaumont 1993). Post settlement selection as an explanation for heterozygote deficiencies in limpets is limited to the pulmonate limpet *Siphonaria jeanae* (Johnson and Black 1982, 1984a).

Samples from both Mallorca and Corsica show overall heterogeneity of allele frequencies. In both cases this is largely due to differences in the *Pgm-1* locus. One possible explanation for this is connected to the proposed Wahlund effect (see above). If the larval supply to a site is from more than one source then the possibility exists that this mixed source will not have the same make up for the different sample sites in Mallorca or Corsica. Thus a

slightly different spectrum of allele frequencies at the *Pgm-1* locus could be supplied to each of the sample sites, resulting in heterogeneity of allele frequencies. Another possible explanation is differential settlement and post-settlement selection (Johnson and Black 1982, 1984a) and thus heterogeneity of allele frequencies.

4.4.2 - Genetic distance and gene flow

The genetic identity values (I) between countries range from 0.852 to 0.983, whilst the lowest I value between any two sites is 0.831. Thorpe (1979, 1982) found that 98% of I values exceeded 0.85 between 1680 conspecific populations studied. The lowest I value falls marginally below this level, when the distances are calculated using UPGMA the level of common identity is 0.88. Although these I values are at the low end of the range for I values of conspecifics, it is still safe to assume that the thirteen samples are all the same species. As even though there are quite low I values between some samples, such as 0.831 between the Ghallis Point and the El Arenal sample, there are samples connecting the two with which both have a higher identity. For example the Genoa sample has an identity of 0.932 with the Ghallis Point sample and 0.898 with the El Arenal sample. Thus the differences between samples are more in the form of clinal variation rather than discrete groups. With the high levels of identity within countries (the lowest being 0.995) it appears that there is a high level of gene flow between the sites from the same country. Similarly, the level of gene flow between all sites in the Eastern Mediterranean Basin is probably very high. The lower identity levels between countries in the Western Mediterranean Basin would tend to suggest restricted gene flow between the countries. It is interesting to note that the sites in Corsica are closer genetically to those in Mallorca than those on the Italian/French mainland, which is geographically much closer. In turn the Italian/French mainland samples are most closely related to the Maltese samples. It is clear that there is some restriction to gene flow between Corsica and the adjacent mainland, but care must be taken in lending too much weight to the genetic associations between countries. The levels of identity between countries are in a narrow range between 0.92 and 0.93 (with the notable exception of between Crete and Cyprus which is higher at 0.98). Quite small variations in allele frequencies within samples could easily affect the I values with respect to which countries are genetically closest to each other. Most of the populations show deviations from Hardy-Weinburg equilibrium, the probable cause (as discussed above) being a Wahlund effect. If the population mixing causing this ef-

fect is stochastic in nature then there could easily be variation in allele frequencies sufficient to change the associations indicated by the I values. Having said this, it is surprising that the Corsican samples are not genetically closer to those from mainland Italy and France. It is possible that currents in the Liguro-Provencal basin, running between Corsica and the mainland act to isolate Corsica from the mainland sites (Pedrotti and Fenaux 1992). Also Pérès (1967), when dividing up the Mediterranean into regions put Genoa and St Tropez in the Northern region of the Western Basin and Corsica and Mallorca both in the Central region. These two regions have different characteristic fauna and flora. The northern region also has no direct Atlantic inflow and experiences winter cooling (Pérès 1967). It is possible that it is not a lack of gene flow between Corsica and the mainland that is causing the differences in allele frequencies but rather different selection pressures acting during settlement and post-settlement in the two regions. Whether this is the case could only really be determined by studies of larval supply and larval genetics. The Atlantic current flow into the Mediterranean passes Mallorca and supplies Corsica (Pérès 1967). The genetic identity between samples from Mallorca and Corsica suggest intermittent gene flow between the two locations. This could be facilitated by a small amount of larval exchange between the two countries when favourable current and weather conditions coincide with a spawning event.

There appears to be a degree of genetic separation between the limpets from the Eastern and the Western Mediterranean basins. The differences are not sufficient, however, for the *P. caerulea* from the Eastern Basin to be regarded as a different species to those in the Western Basin. There would still appear to be some intermittent exchange of larvae between the two basins.

The levels of gene flow found in this study are lower than those found in studies of gene flow around the Italian coast and Corsica (Sella *et al.* 1985; Badino *et al.* 1985). These previous studies only included three loci one of which (*Mdh-1*) was monomorphic (by the 95% criterion) in nine out of the eleven samples a second loci (*Pgm-1*) did not include data for Corsica. Thus it is difficult to compare the studies with this one which is essentially looking at gene flow between the islands of the Mediterranean.

4.4.3 - Morphology

The genetic data shows a strong relationship between sites from the same country, and particularly in the Eastern Mediterranean Basin regional similarities in the allele frequencies of samples. This is not reflected in the shell morphology of the samples. Although there are some similarities between limpets collected in the same country, most notably Corsica and Malta, these associations are not very strong compared with the genetic associations. There is a high degree of overlap in shell shape between samples. Both within a sample and over the data set as a whole, there is a wide variation in shell shape of *P. caerulea*. This is probably due to the fact that *P. caerulea* is opportunistic and exploits a wide variety of microhabitats on any shore and that there is a strong environmental component influencing shell shape. If there are any shell shape characteristics that are specific to a country or region they are obscured by the high overall level of variation in shape. It is most likely that similarities in shell shape between sites are due to the sites having similar environmental conditions (such as level of exposure, rock type etc.). Indeed the two sites in Corsica (which show a relationship in the canonical variate analysis) are of similar aspect and exposure and were the only two sites to be based on a granite bedrock. Also the other three sites that show a close association from the canonical variate analysis: Ghallis Point, Xghajra and Mal Pas are all natural shores rather than breakwaters, and all of a similar soft rock type.

There was a wide range of shell sizes in the samples with the Ghallis Point sample having the smallest mean size for each of the various shell parameters. The average size of individuals at a site may be influenced by environmental conditions such as food supply and rock type. It was noticed that at all the sites with a soft rock type (Ghallis Point, Xghajra and Mal Pas) all the limpets appear to have a mean size lower than the average for the data set as a whole (see principal component analysis results). The home scars of limpets at these sites were very deep relative to the size of the limpet (in some cases the apex of the shell was at the same height as the surrounding rock). This could possibly restrict the growth of the shell. A second factor influencing the mean shell size is the collection of limpets by man for food and fishing bait. The Maltese sites were heavily exploited in this way and large individuals of *P. caerulea* were only found on inaccessible areas of the shore.

4.4.4 - Summary

The *P.caerulea* samples from the Mediterranean can viewed as constituting a single species. The genetic variation between limpets throughout the Mediterranean seems to be clinal, although there appears to be some hydrographical barriers to gene flow, notably between Corsica and Northern Italy and between the eastern and western basins. It is probable that intermittent genetic exchange prevents divergence of the limpets from the two basins. The heterozygote deficiencies found in the samples suggest that there are complex interactions in the larval distribution and settlement of this species that affect the genetic make up of poppulations. The species shows high levels of intra-specific morphological variation, the differences in morphology are probably not dictated by geographical location but rather by habitat differences between the sample sites.

5 - *P. caerulea* and *P. depressa* - the separation of two morphologically similar patellids

5.1 - Introduction

P. caerulea is the dominant mesolittoral limpet on moderately exposed rocky shores in the Mediterranean (Davies 1969; Sella and Bacci 1971; Bannister 1975; Sella 1976; Della Santina and Chelazzi 1991). Geographically its distribution extends from Israel (Lavie *et al.* 1987) in the east to Tangiers (Morocco) and to Tarifa (Spain) in the west (Fischer-Piette & Gaillard, 1959). *P. depressa* is the analogous mid-shore species on the Atlantic coasts of Southern Europe and Northern Africa (Fretter and Graham 1976). *P. depressa* has a possible southern limit of Senegal (Pilsbry 1891) and a northern limit on the European mainland coast at Lîle Saint-Marcouf in France (Fischer-Piette 1941); additionally it is found in the British Isles as far as Anglesey in Britain (Crisp and Knight-Jones 1955; Lewis 1964; Fretter and Graham 1976). *P. depressa* has not been found in Ireland or the Atlantic Islands of the Canaries, Maderia and the Azores (Purchon 1968; Hawkins *et al.* 1990; Côrte-Real 1992). *P. depressa* also extends a short distance into the Mediterranean along the North African and Spanish coasts (Christaens 1973). Grandfil and Vega (1982) have recorded *P. depressa* on the Spanish Mediterranean coast at Nerja. Disputing earlier records (Morton 1967) recent work suggests that *P. caerulea* is not present in the Azores (Christiaens 1973; Hawkins *et al.* 1990).

There is clearly a small overlap in distribution between these two species. Where *P. depressa* has extended into the Mediterranean on the Spanish coast it can be difficult to distinguish from *P. caerulea* (Côrte-Real 1992; Hawkins *pers. comm.*). Both species can have white pallial tentacles (for *P. depressa* see Evans 1947 ; for *P. caerulea* see Sella 1976) and similar shell morphology. When Côrte-Real genetically analysed *P. depressa* and *P. caerulea* from Estopona, Spain it was evident that the *P. depressa* sample also contained *P. caerulea* . Indeed subsequent re-examination of the *P. depressa* sample from Estopona suggested that the vast majority of the individuals identified as *P. depressa* were in fact *P. caerulea* (Côrte-Real *pers. comm.*).

With doubt as to the true extent of the penetration of *P. depressa* into the Mediterranean and the strong morphological similarities between the supposed Mediterranean populations of *P. depressa* and the endemic *P. caerulea* the relationship between these two species is in need of investigation, and differences between the two species need highlighting.

This chapter will compare the morphological and biochemical genetic differences between *P. depressa* and *P. caerulea* with a number of objectives in mind.

1 - Finding diagnostic aids for distinguishing the two species where they are sympatric. It is hoped that the use of biochemical genetics will reveal diagnostic genetic loci that will subsequently allow the extent to which *P. depressa* extends into the Mediterranean to be assessed in the future. The work will try to establish strong morphological and genetic characters for the separation of *P. depressa* and *P. caerulea* along the Spanish Mediterranean coast where they are sympatric.

2 - Confirm with the aid of biochemical genetics that the two species are indeed different, rather than being the Atlantic (*P. depressa*) and Mediterranean (*P. caerulea*) form of the same species.

3 - Compare the *P. depressa* from Morocco and Portugal to see whether the Atlantic inflow into the Mediterranean acts as a barrier to gene flow between the two sides of the mouth of the Mediterranean.

4 - Use assessments of levels of gene flow between sites to attempt to explain the limits to geographic distribution of the two species. Is it the current patterns in the Alboran Sea that prevent *P. caerulea* extending into the Atlantic? What prevents *P. depressa* extending into the Mediterranean on a large scale?

The samples of *P. caerulea* used are taken from a range of sites throughout the Mediterranean where *P. depressa* has not been reported. The *P. depressa* samples are from: Estoril (Portugal) where *P. caerulea* does not occur; and Tangiers and Jbel Musa in Morocco where the *P. depressa* found have morphology similar to those found in Estoril and clearly conform to the *P. depressa* morphotype.

5.2 - Materials and methods

5.2.1 - Collection of samples

Samples of *P. caerulea* were collected from six sites spread throughout the Mediterranean: El Arenal (Mallorca), Calvi (Corsica); Genoa (Italy); Ghallis Point (Malta); Irepetra (Crete) and Limassol (Cyprus) (See figure 2.1 & 2.2). At each site a sample of approximately 100 individuals was taken in the mesolittoral zone from above the red algal turf zone that marks the top of the infralittoral fringe. Samples of *P. depressa* were collected from two sites in Morocco (Tangiers Breakwater and Jbel Musa) and from one site in Portugal (Estoril). All three samples were collected from the mid-shore zone.

5.2.2 - Qualitative soft body characters

A number of soft body characteristics were recorded for each limpet: colour of head tentacles, colour of pallial tentacles, presence of orange base to pallial tentacles, and colour of the foot muscle. The sex , gonad stage and colour of each individual was also recorded (following Orton 1956) .

5.2.3 - Morphological analysis of shell

For each limpet a number of shell parameters were measured, to the nearest 0.5mm using vernier callipers (Figure 2.3). The two width measurements give an impression of how ovate the shell is. Differences between the two height measures indicate curvature in the basal plane of the shell. Additionally the shell was weighed to the nearest 0.01g. Shells were labelled and stored for future reference.

The nine samples (Estoril, Tangiers, Jbel Musa, El Arenal, Calvi, Genoa, Ghallis Point, Irepetra and Limassol) were treated separately in the analysis of the data. Additionally the data were grouped by species (i.e. *P. depressa* and *P. caerulea* as the two groups). The three major techniques used in the analysis of the data were: principal component analysis,

canonical variate analysis and linear discriminant function analysis. Further details of these techniques are given in chapter 2.

For this study the radula length of each individual was not used, there were two reasons for this:

1) If the study came up with shell characteristics that strongly differentiated the samples of *P. depressa* from the samples of *P. caerulea*, this information could then be used to re-examine at museum shell collections. It is unlikely that such shell collections will contain information about the radula length.

2) It allowed the inclusion of the *P. depressa* sample from Jbel Musa where there was no information about the radula length of individuals.

From chapter 2 it is clear that there is little loss of discrimination between groups of *P. caerulea* where radula length is not included, so the exclusion of this character will still allow differentiation between groups based on the remaining seven shell characters.

As relative radula length (R/C ratio) is often used to differentiate species (Fischer-Piette 1935; Fischer-Piette and Gaillard 1959; Christiaens 1973; Bannister 1975) the analysis was repeated with the radula length of each individual used in addition to the shell measures. This necessitated the exclusion of the Jbel Musa sample so only two *P. depressa* samples could be used. This second analysis was used to see whether radula length was important in the morphological differentiation of *P. depressa* and *P. caerulea*.

5.2.4 - Electrophoresis

Electrophoresis on the samples was carried out following the methods described in detail in the general materials and methods (Chapter 2). In brief, for the electrophoretic work the foot muscle tissue of the limpet was used. The muscle was removed from live limpets; only healthy individuals were sampled. The genetics of the samples of limpets was examined using standard horizontal starch gel electrophoresis (following Selander *et al.* 1971; Harris and Hopkinson 1977; Ferguson 1980). Tris citrate pH 8.0 was used as the gel buffer. The gel slices were stained using standard solutions (Shaw and Prasad, 1970; Schaal and Ander-

son, 1974; Harris and Hopkinson, 1978). Thirteen loci were assayed : *Pgi-1*, *Pgm-1*, *G3pdh*, *Pgdh*, *Np*, *Mpi-1*, *Mdh-1*, *Mdh-2*, *Ald-1*, *Idh-1*, *Idh-2*, *Me-2* and *Got*.

The analysis of electrophoretic results was based on the genotype frequencies of the loci for each species at each site. Genetic statistics were calculated using the program BIOSYS-1 Release 1.7 (Swofford and Selender, 1989).

5.3 - Results

As the variation in shell shape and genetics of *P. caerulea* has already been examined in detail in the previous chapter, this study will concentrate on differences between *P. caerulea* and *P. depressa* and look at variation within *P. depressa*.

5.3.1 - Soft body characteristics

There is a high degree of variation in the soft body characteristics of *P. caerulea*. The foot generally varies in colour between a light cream and a very dark grey/black and generally the central region of the foot is a lighter colour than the outer edge, varying between a light cream and a dark orange. The colour of the head tentacles of *P. caerulea* varies from a dark grey/black through grey to translucent. The presence of an orange base to the pallial tentacles and pallial tentacles of a stark white colour varies between and within samples.

The foot colour of *P. depressa* varied between cream and a dark grey and as in *P. caerulea* most individuals had a light colour in the centre of the foot, varying between cream and a dark orange. *P. depressa* did show more individuals with a orange/brown foot colour but this coloration was also found in *P. caerulea*. As in *P. caerulea* the head tentacle colour of *P. depressa* varied between a dark grey/black to translucent, though a higher proportion of *P. depressa* had grey tentacles than did *P. caerulea*. All of the *P. depressa* had stark white pallial tentacles and none had the orange base to the tentacles found in some *P. caerulea*. However, as all samples of *P. caerulea* contained individuals with stark white tentacles this feature cannot be used to distinguish the two species.

Generally there were no soft body characters that could be used definitively to separate *P. depressa* from *P. caerulea* as both species showed a wide range of variation for these characters. The variation in *P. caerulea* was greater than that found for *P. depressa*.

5.3.2 - General morphological results

Table 5.1 gives the correlation coefficients between the $\log_{10}(x+1)$ values of the seven shell parameters used. The degree of correlation between characters is lower than those found in the analysis of variation in a single species (See Chapter 4, Table 4.1). This is due to the fact that the analysis covers two species: *P. caerulea* and *P. depressa*. If there are different associations between the shell characters for the two species then combined shell data for the two species will obviously give a relationship with more noise and a lower correlation coefficient. One would intuitively expect lower correlation coefficients if the two species have differing isometric shell shape relationships.

Table 5.1 - Correlation coefficients for data comparing the three *P. depressa* samples and the six *P. caerulea* samples. Data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize variance. Note that Radula length is not included in the analysis.

	Length	Width (max)	Height (width)	Heightl (length)	Apex	Width (apex)	Cube root of weigth
Length	1.000						
Width (max)	0.952	1.000					
Height (width)	0.814	0.812	1.000				
Height (length)	0.829	0.806	0.980	1.000			
Apex	0.840	0.809	0.683	0.720	1.000		
Width (apex)	0.941	0.969	0.816	0.814	0.846	1.000	
Cube root of weight	0.920	0.905	0.915	0.921	0.742	0.897	1.000

Table 5.2 - Eigenvalues for data comparing the three *P. depressa* samples and the six *P. caerulea* samples. Data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize variance. Note that radula length is not included in the analysis.

	Eigenvalue	Percentage of variance	Cumulative percentage
Component 1	6.132	87.6	87.6
Component 2	0.483	6.9	94.5
Component 3	0.229	3.2	97.8
Component 4	0.075	1.1	98.8
Component 5	0.041	0.6	99.4
Component 6	0.026	0.4	99.8
Component 7	0.014	0.2	100.0

5.3.3 - Principal component analysis

The first principal component in the analysis accounts for 87.6% of the variance in the data set (Table 5.2). There are approximately equal weightings for all of the elements in the eigenvector of principal component 1 (Table 5.3), the value of each element being approximately equal to the predicted value for an isometric size vector $p^{-1/2} = 0.378$ (Jolicoeur, 1963). Principal component 1 can therefore be regarded as an isometric size vector, thus 87.6% of the variance in the data set is due to isometric variation in shell size. Where the data set was broken down into samples of the two species, then the *P. depressa* sample has a significantly larger mean shell size than the *P. caerulea* sample ($F=169.84$, $p<0.001$). A frequency distribution of individuals from the two species along principal component 1 (Fig 5.1) reflects this difference in mean size. The distributions of the samples from both species along this axis approximated to normality. Despite the significantly larger mean size in *P. depressa* there is a good deal of overlap in the size frequency distributions of the two species. Looking at the individual sites (Fig 5.2) the samples of *P. depressa* from Jbel Musa and Estoril have the largest mean size, with Tangiers having the fourth largest mean shell size. The sample from Jbel Musa is significantly larger than all the other samples. The Ghallis Point sample has a significantly smaller mean shell size than all the other samples, with the Limassol sample also having a small mean shell size when compared with the other samples.

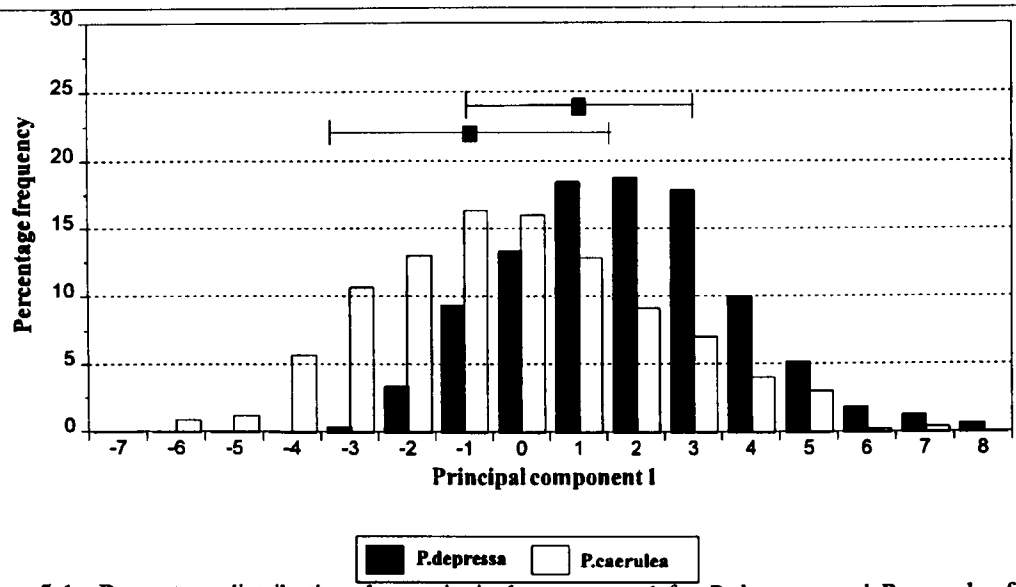


Figure 5.1 - Percentage distribution along principal component 1 for *P.depressa* and *P.caerulea* for combined samples. The data have been $\log_{10}(x+1)$ transformed and standardized. The data are separated by species, with group mean and 95% confidence interval of the population shown. There are significant differences between groups ($F=169.84$, $p<0.001$).

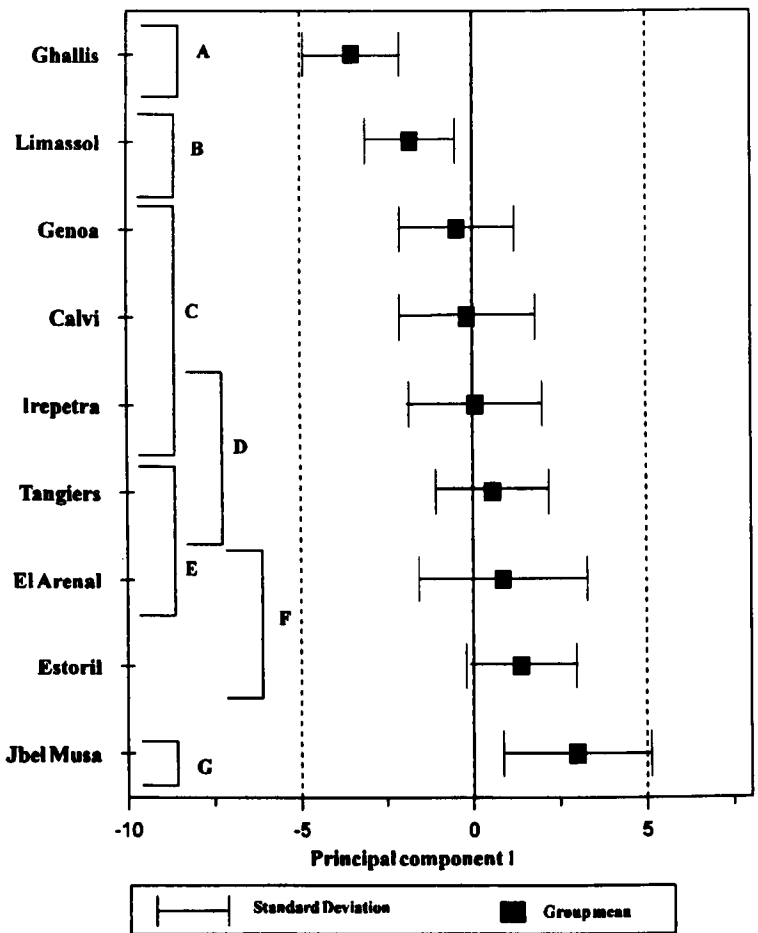


Figure 5.2 - Distribution of the group means of the three *P.depressa* samples and the six *P.caerulea* samples along principal component 1, with standard deviations shown. There are significant differences between groups ($F=90.46$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data.

Table 5.3 - Eigenvectors for data comparing the three *P. depressa* samples and the six *P. caerulea* samples. Data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize variance. $p^{-1/2}$ is the predicted score for each element in principal component 1 if principal component 1 is an isometric size vector. Note that radula length is not included in the analysis.

	Principal component 1	Principal component 2	Principal component 3	Principal component 4
% of variance	87.6	6.9	3.2	1.1
Length	0.388	0.215	-0.231	-0.543
Width (max)	0.386	0.231	-0.417	0.300
Height (width)	0.371	-0.524	0.171	0.301
Height (length)	0.374	-0.482	0.293	0.005
Apex	0.346	0.530	0.742	-0.057
Width (apex)	0.388	0.258	-0.241	0.512
Cube root of weight	0.389	-0.210	-0.227	-0.508
$p^{-1/2}$	0.378			

The second principal component accounts for 6.9% of the variance in the data set (Table 5.3). This component is mainly a contrast between shell height (both shell measures being of approximately equal magnitude and sign) and the anterior to apex distance (i.e. how far to the posterior of the shell the apex is). From the distribution of sample group means along principal component 2 (Fig 5.3), this component separates the three *P. depressa* samples from the six *P. caerulea* samples. With low group means individuals of *P. depressa*, when compared with *P. caerulea* of the same size, will have a greater shell height and an apex nearer the anterior of the shell. This result is reflected in an ANOVA based on the group means of the two species along principal component 2 which gives a highly significant result ($F=446.14$ $p<0.001$) with the *P. depressa* sample having a lower mean (-0.537) than the *P. caerulea* sample (0.296) (Fig 5.4). Although the frequency distributions overlap there is a clear separation of the two species along this axis. In the principal component analysis it is this second principal component that mainly acts to separate the two species.

Principal component 3 accounts for 3.2% of the data set variance (Table 5.3), and is dominated by maximum shell width and anterior to apex measures, with a negative relationship between the two shell parameters. Although the group means for the two species are significantly different from each other ($F=92.52$, $p<0.001$) with a mean of 0.215 for *P. depressa* and 0.118 for *P. caerulea*, the frequency distribution along this axis shows there

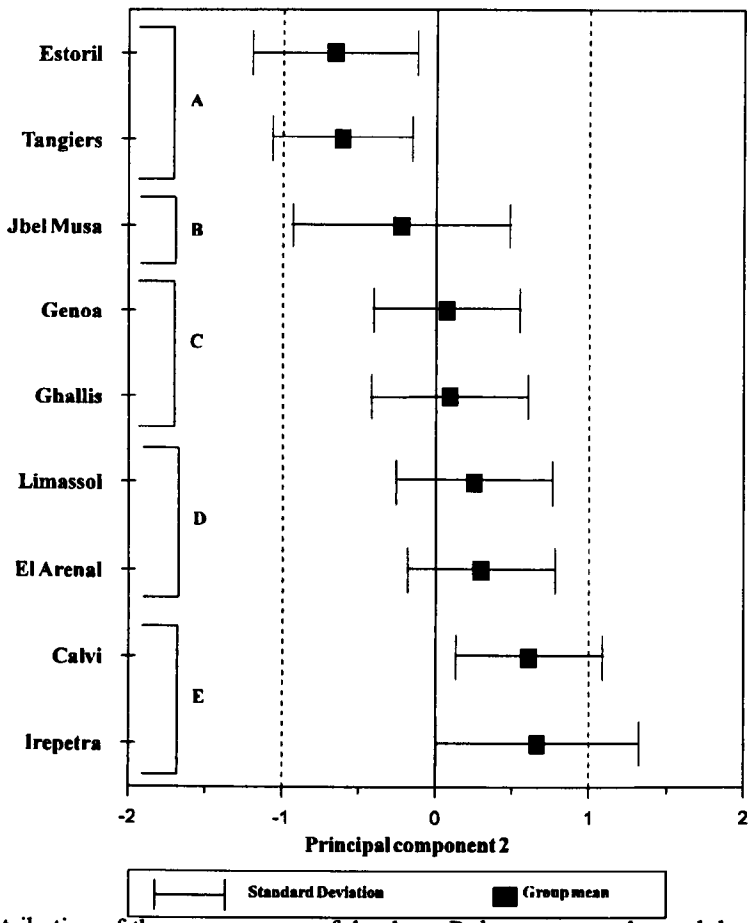


Figure 5.3 - Distribution of the group means of the three *P.depressa* samples and the six *P.caerulea* samples along principal component 2, with standard deviations shown. There are significant differences between groups ($F=90.26$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data.

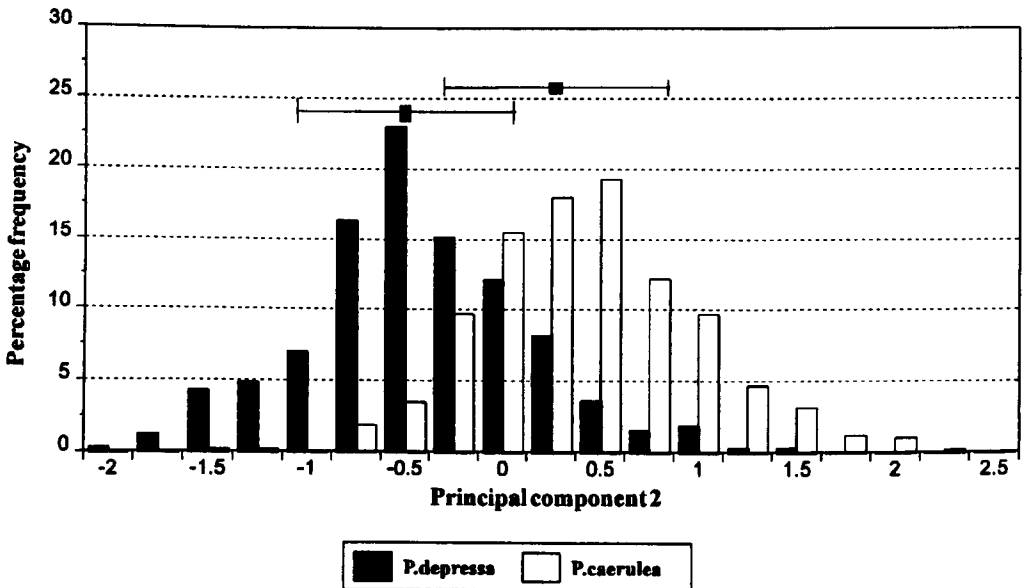


Figure 5.4 - Percentage distribution along principal component 2 for *P.depressa* and *P.caerulea* for combined samples. The data have been $\log_{10}(x+1)$ transformed and standardized. The data are separated by species, with group mean and 95% confidence interval of the population shown. There are significant differences between groups ($F=446.14$, $p<0.001$).

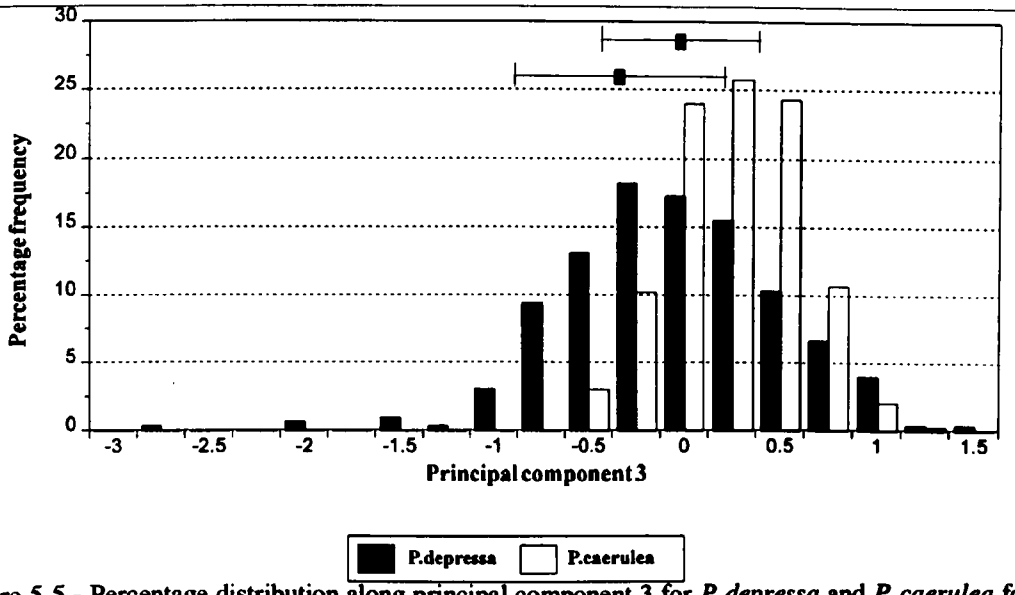


Figure 5.5 - Percentage distribution along principal component 3 for *P.depressa* and *P.caerulea* for combined samples. The data have been $\log_{10}(x+1)$ transformed and standardized. The data are separated by species, with group mean and 95% confidence interval of the population shown. There are significant differences between groups ($F=92.52$, $p<0.001$).

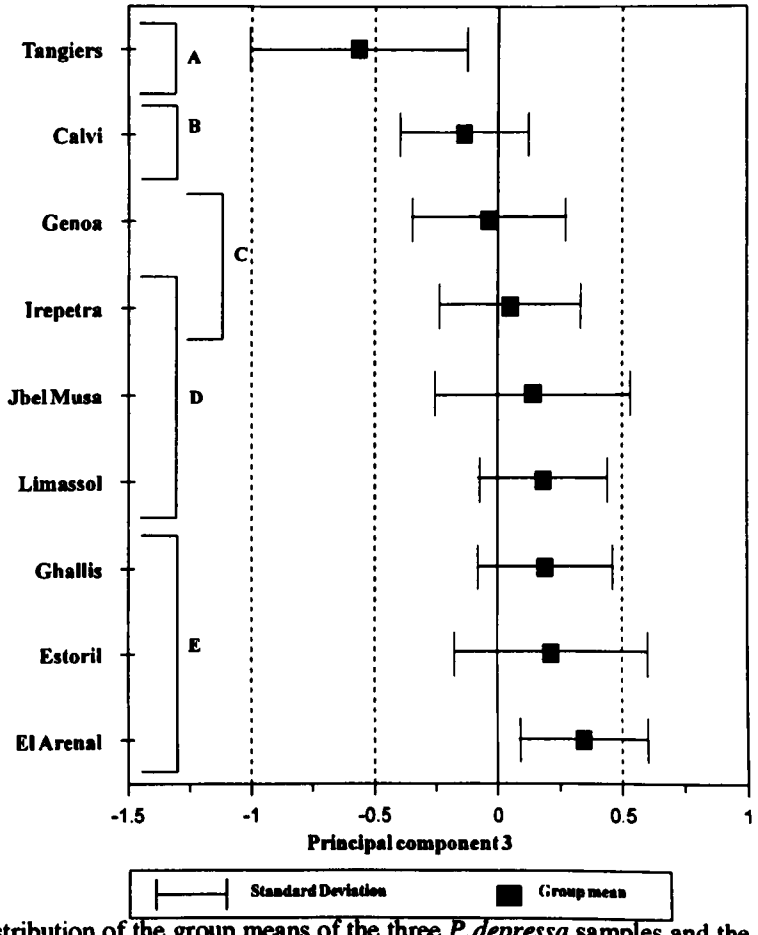


Figure 5.6 - Distribution of the group means of the three *P.depressa* samples and the six *P.caerulea* samples along principal component 3, with standard deviations shown. There are significant differences between groups ($F=65.60$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data.

to be a large overlap in the distributions (Fig 5.5). Looking at the distribution of the group means of the nine sites (Fig 5.6) along the third principal component it is clear that the difference in group means between the samples from the two countries is largely due to the Tangiers sample of *P. depressa*. The mean of the Tangiers sample is well separated from the other sample group means. Individuals from Tangiers have a greater maximum shell width and a shell apex closer to the anterior of the shell than do individuals from the other samples, including the other *P. depressa* samples. The majority of other groups do not show significant differences from each other along the third principal axis. Clearly it is this third axis that acts to differentiate the Tangiers sample from the other two *P. depressa* samples. It is worth noting at this juncture that of the *P. depressa* samples the Tangiers sample is the only one collected from a breakwater. Breakwaters tend to offer a wider variety of microhabitats and thus between this sample and the other two *P. depressa* samples differences in shell shape are to be expected.

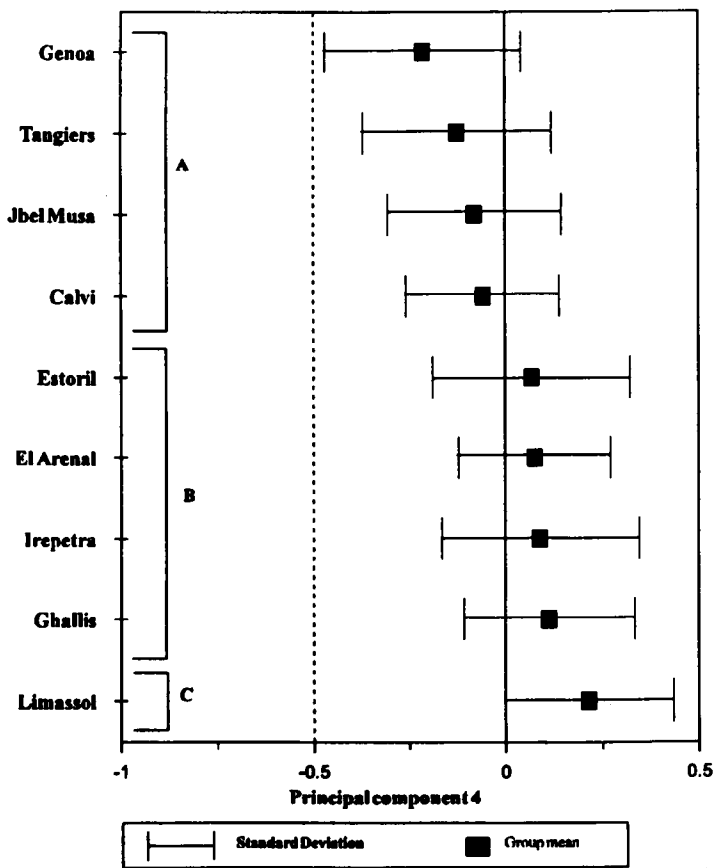


Figure 5.7 - Distribution of the group means of the three *P.depressa* samples and the six *P.caerulea* samples along principal component 4, with standard deviations shown. There are significant differences between groups ($F = 19.30$, $p < 0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data.

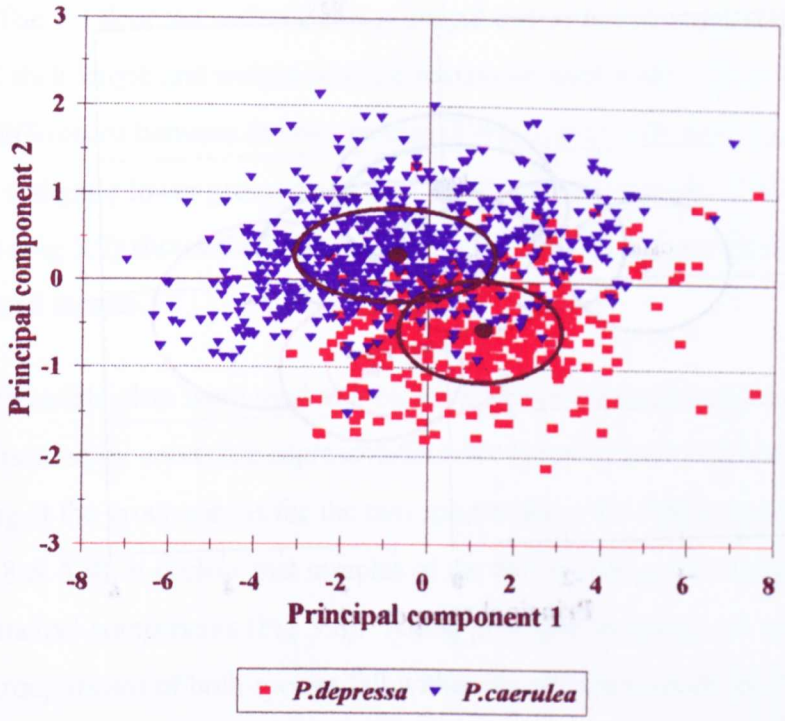


Figure 5.8 - Plot of principal component 1 against principal component 2 for the three *P.depressa* samples and the six *P.caerulea* samples. The data are differentiated by species. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data. The data are separated by site, with group centroids and standard deviations shown.

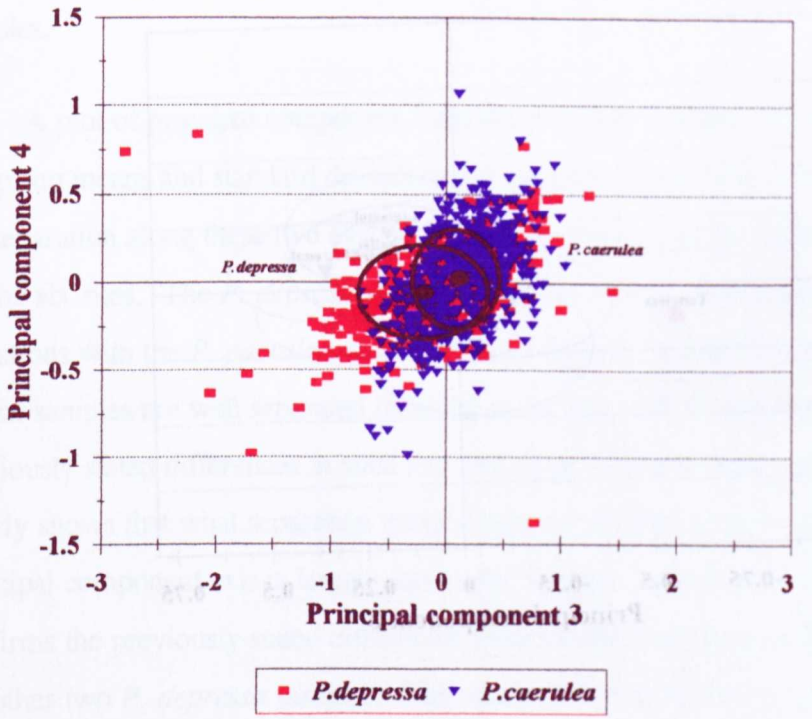


Figure 5.9 - Plot of principal component 3 against principal component 4 for the three *P.depressa* samples and the six *P.caerulea* samples. The data are differentiated by species. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data. The data are separated by site, with group centroids and standard deviations shown.

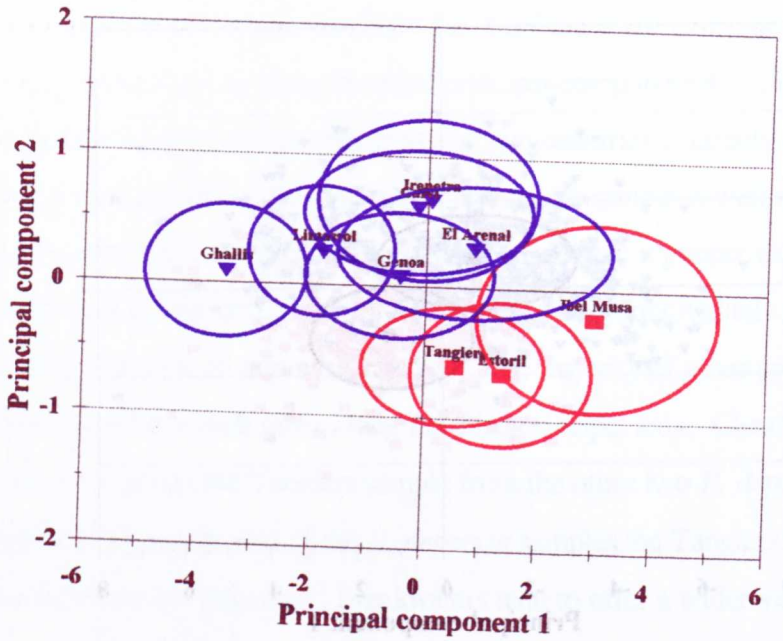


Figure 5.10 - Plot of principal component 1 against principal component 2 for the three *P.depressa* samples and the six *P.caerulea* samples. The data are differentiated by sample site. The data have been log10(x+1) transformed and a correlation matrix used to standardise the data. The data are separated by site, with group centroids and standard deviations shown.

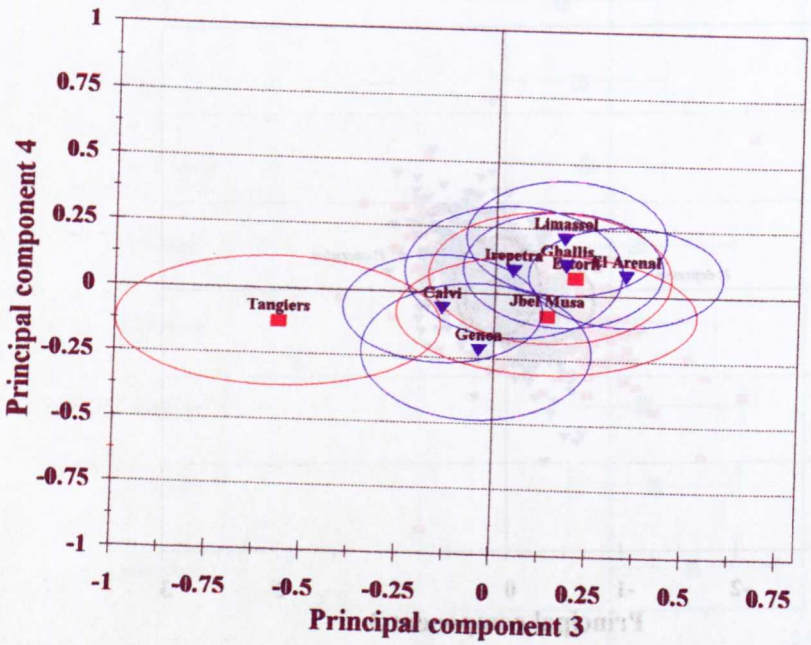


Figure 5.11 - Plot of principal component 3 against principal component 4 for the three *P.depressa* samples and the six *P.caerulea* samples. The data are differentiated by sample site. The data have been log10(x+1) transformed and a correlation matrix used to standardise the data. The data are separated by site, with group centroids and standard deviations shown.

The 1.1% of the variance that principal component 4 accounts for (Table 5.3) is a contrast of shell length and weight with the maximum shell width. This component shows slight differences between the two species ($F=8.2$, $p<0.01$) with the *P. depressa* sample having a slightly lower group mean than the *P. caerulea* sample. The differentiation by sample site (Fig 5.7) shows the Tangiers and Jbel Musa samples are significantly different from the Estoril sample.

Bivariate plots were used to look for differentiation between the species or individual sites obscured by univariate representations for the principal component analysis results. Looking at the group means for the two species along the first four principal components (Fig 5.8 & 5.9), it is clear that samples of the two species are best separated along the first two principal components (Fig 5.8). Along principal component 3 and principal component 4 the group means of both species fall within the standard deviation of the mean for the other species (Fig 5.9). The differentiation along principal component 1 and principal component 2 (Fig 5.8) indicate that the *P. depressa* samples show a larger mean size coupled with a relatively taller shell and a shell apex closer to the anterior of the shell than do the *P. caerulea* samples.

A plot of principal component 1 against principal component 2 with the individual sample group means and standard deviations for each site shown (Fig 5.10) clearly shows that the separation along these two axis of the two species is strongly reflected in the group means for the six sites. The *P. depressa* samples from Jbel Musa show a larger overlap of standard deviations with the *P. caerulea* sample from El Arenal. Apart from that the three *P. depressa* samples are well separated from the six samples of *P. caerulea*, thus confirming the previously stated differences in shell size and shape between these two species. Figure 5.11 clearly shows that what separation there is between the two species along the third and fourth principal component axis is largely due to the Tangiers sample of *P. depressa*. This again confirms the previously stated differences between the Tangiers sample of *P. depressa* and the other two *P. depressa* samples. The standard deviations for the groups are not shown as, with the exception of the Tangiers sample, they all overlap heavily.

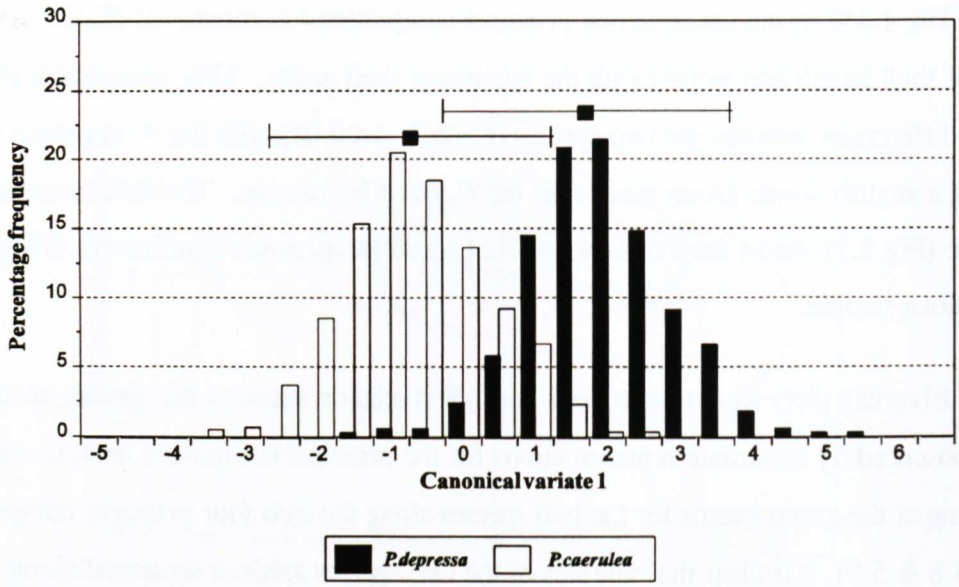


Figure 5.12 - Percentage distribution along canonical variate 1 for P.depressa and P.caerulea for combined samples. The data have been log10(x+1) transformed and standardized. The data are separated by species, with group centroids and 95% confidence intervals shown.

5.3.4- Canonical Discriminant Analysis and Cross-validated Discriminant Function Analysis

Using canonical variate analysis to look at the differences between the two species (*P. depressa* and *P. caerulea*), the analysis produces a single canonical variate since the maximum dimensionality for a canonical variate representation is **s**, where:

s = min(**p**,**g**-1)

p = number of variables

g = number of groups.

The canonical variate is mainly a contrast of the shell weight with the apex to anterior measure, with contributions from the shell height (lengthways) and a negative width across apex measure (Table 5.4). The distribution of individuals from the two species along the canonical variate axis are shown in figure 5.12. There is a clear separation of the two species along this axis, with the *P. depressa* sample having a larger mean value than the *P. caerulea* sample. The confidence limits shown are the limits within which 95% of the populations are predicted to be found (1.960 either side of the mean). The 95% confidence limits of the mean for *P. depressa* and *P. caerulea* are 0.108 and 0.080 respectively. Based on the ca-

nonical variate coefficients the difference between the two species are a greater shell weight and a more anterial apex coupled with a slightly greater shell height and a smaller width across the shell apex in *P. depressa* than in *P. caerulea*. The cross tabulated linear discriminant function analysis reflects this high degree of separation between the two species (Table 5.5) with 91.24% of *P. depressa* and 88.69% of *P. caerulea* being assigned to the correct group based on the linear discriminant function generated.

Table 5.4 - Standardized canonical coefficients for the *P.depressa* samples and the *P.caerulea* samples differentiated by species. The data have been log10(x + 1) transformed and standardized. Note that radula length is not included in the analysis.

Measured character	Canonical variate 1
Length	-0.423
Width (max)	0.219
Height (width)	0.208
Height (length)	0.684
Apex	-1.331
Width (apex)	-0.614
Cube root of weight	1.989

Table 5.5 - Linear discriminant function using cross-validation showing percentage of individuals from the *P. depressa* samples and the *P. caerulea* samples correctly assigned to their original groups (bold). The data are differentiated by species.

Site	*P. depressa*	*P. caerulea*
P. depressa	**91.24**	8.76
P. caerulea	11.31	**88.69**
Percentage allocated to group	39.70	60.30
Percentage of original data set	35.52	64.48

The canonical variate analysis and linear discriminant function analysis was repeated after breaking the data set down into the samples from the nine sites. The first four canonical variates account for 95% of the variance in the data set (Table 5.6) with the first canonical variate accounting for 57.6% of the variance. The pairwise squared distances between the nine groups are given in table 5.7. The closest two groups are the two *P. depressa* samples from Estoril and Jbel Musa (1.750) whilst the greatest squared distance between any two sites is 22.15, between *P. depressa* from Tangiers and *P. caerulea* from Ghallis Point. The squared distances were used to produce a dendrogram based on a UPGMA cluster analysis

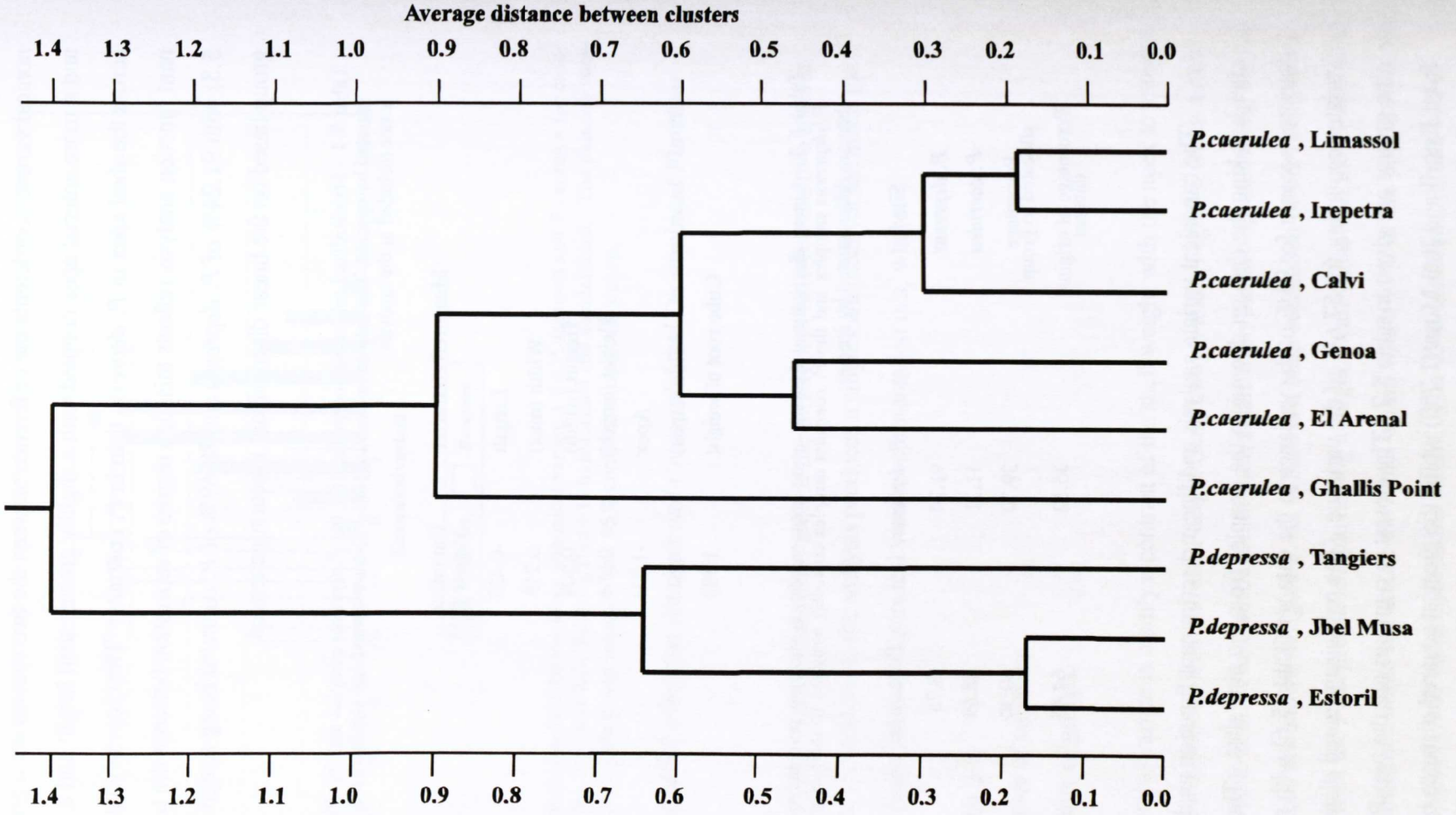


Figure 5.13 - Dendrogram based on an UPGMA cluster analysis of the pairwise squared canonical distances between the three samples of *P. depressa* and the six samples of *P. caerulea*.

Table 5.7 - Canonical pairwise squared distances between groups for the three *P.depressa* samples and the six *P.caerulea* samples differentiated by site. The data have been $\log_{10}(x+1)$ transformed and standardized. Note that radula length is not included in the analysis.

Site	*P.depressa* Estoril	*P.depressa* Tangiers	*P.depressa* Jbel Musa	*P.caerulea* El Arenal	*P.caerulea* Calvi	*P.caerulea* Genoa	*P.caerulea* Ghallis Point	*P.caerulea* Irepetra	*P.caerulea* Limassol
P.depressa **Estoril**	0								
P.depressa **Tangiers**	6.057	0							
P.depressa **Jbel Musa**	1.750	6.699	0						
P.caerulea **El Arenal**	7.370	14.583	6.558	0					
P.caerulea **Calvi**	14.182	12.908	13.382	6.777	0				
P.caerulea **Genoa**	4.807	6.486	4.888	4.333	5.150	0			
P.caerulea **Ghallis Point**	17.961	22.149	21.528	10.897	11.958	9.806	0		
P.caerulea **Irepetra**	14.189	15.973	13.230	4.157	2.102	6.212	7.037		
P.caerulea **Limassol**	14.783	18.104	17.190	5.934	3.659	7.417	4.224	1.819	0

(Fig 5.13). The separation between the two species is reflected in the dendrogram. Although the Tangiers sample is noticeably different from the other two *P. depressa* samples and the Ghallis Point sample is noticeably different from the other *P. caerulea* samples, the three *P. depressa* samples are very clearly separated from the six *P. caerulea* samples. If this dendrogram is compared with that in chapter 4 for the thirteen *P. caerulea* samples (Fig 4.10) the groupings of the *P. caerulea* samples is different. This difference is probably due to the fact that radula length is not being used in this analysis.

Table 5.6 - Standardized canonical coefficients for the three *P. depressa* samples and the six *P. caerulea* samples differentiated by site. The data have been $\log_{10}(x+1)$ transformed and standardized. Note that radula length is not included in the analysis.

Measured character	Canonical variate 1	Canonical variate 2	Canonical variate 3	Canonical variate 4	Canonical variate 5
Percentage of variance	57.6	19.9	14.6	3.3	2.6
Length	0.817	0.376	-2.957	-2.088	-0.376
Width (max)	0.278	-1.001	-1.204	2.529	-0.346
Height (width)	-0.524	-2.677	-1.934	1.092	-4.317
Height (length)	1.275	3.510	1.810	1.026	4.345
Apex	-1.783	1.323	0.332	0.074	-1.489
Width (apex)	-1.131	-0.202	0.850	-0.176	3.111
Cube root of weight	1.985	-0.466	2.539	-2.144	-1.105

Table 5.8 - Group means of the first five canonical variates for the three *P. depressa* samples and the six *P. caerulea* samples differentiated by site. The data have been $\log_{10}(x+1)$ transformed and standardized. Note that radula length is not included in the analysis.

SITE	Canonical variate 1	Canonical variate 2	Canonical variate 3	Canonical variate 4	Canonical variate 5
P. depressa Estoril	1.542	0.858	0.739	0.300	-0.652
P. depressa Tangiers	2.159	-1.186	-0.020	0.222	0.303
P. depressa Jbel Musa	1.677	1.200	-0.001	-0.121	-0.056
P. caerulea El Arenal	-0.637	1.388	-0.306	0.121	0.436
P. caerulea Calvi	-0.969	-0.701	-1.521	-0.184	-0.369
P. caerulea Genoa	0.253	-0.031	0.059	-0.942	-0.074
P. caerulea Ghallis Point	-2.180	-0.685	1.675	-0.108	0.137
P. caerulea Irepetra	-1.566	-0.144	-0.776	0.296	0.243
P. caerulea Limassol	-1.951	-0.428	-0.119	0.562	-0.385

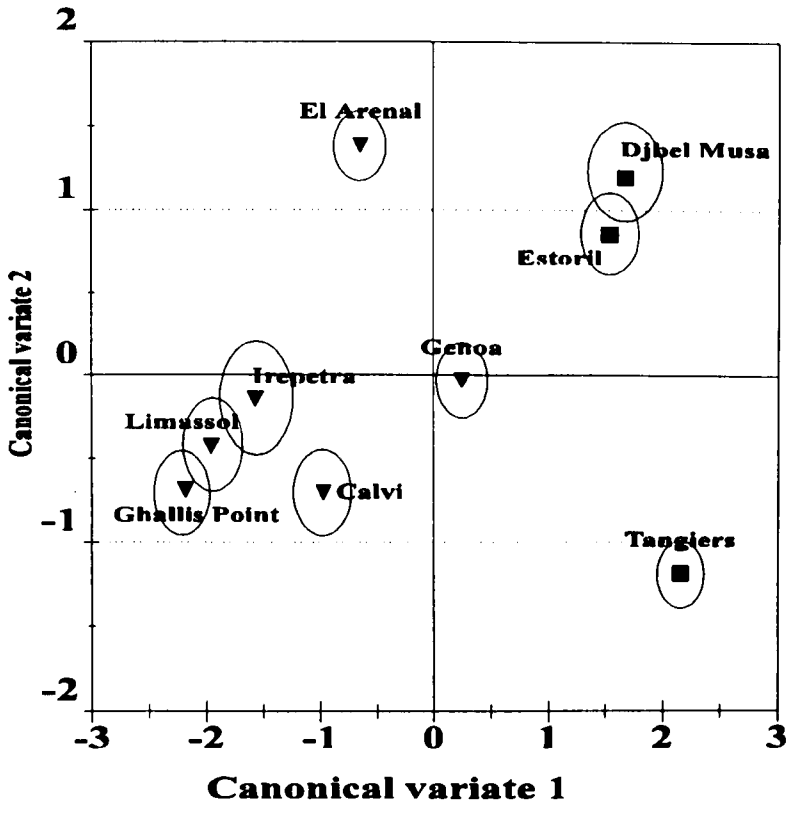


Figure 5.14 - Plot of canonical variate 1 against canonical variate 2 for the three *P.depressa* (■)samples and the six *P.caerulea* (▽) samples. The data have been $\log_{10}(x+1)$ transformed and standardized. The data are separated by site, with group centroids and 95% confidence intervals of the mean shown.

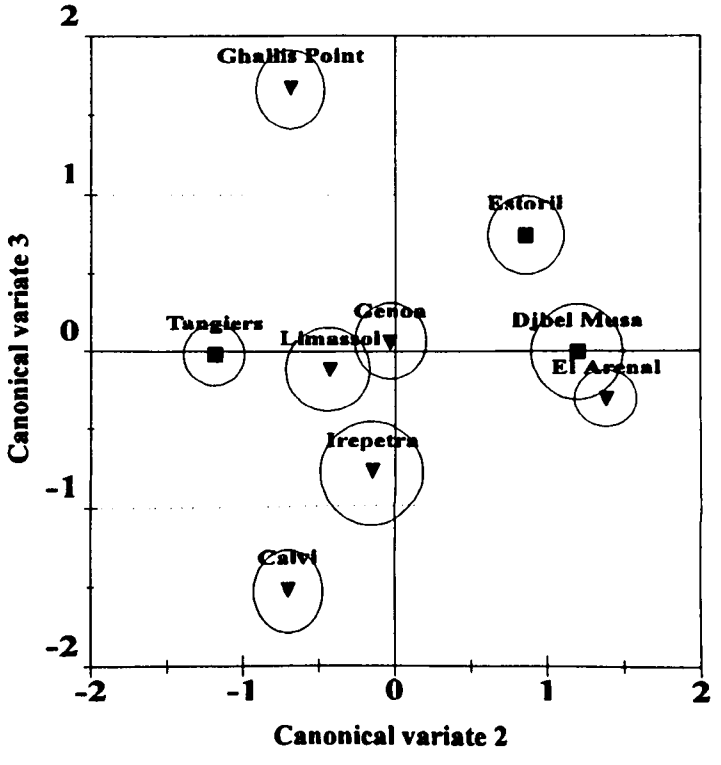


Figure 5.15 - Plot of canonical variate 2 against canonical variate 3 for the three *P.depressa* (■)samples and the six *P.caerulea* (▽) samples. The data have been $\log_{10}(x+1)$ transformed and standardized. The data are separated by site, with group centroids and 95% confidence intervals of the mean shown.

The group means along the first four canonical variate axes (Table 5.8) were used in bivariate plots to see which canonical variates were instrumental in separating the groups from the two species and hence which shell characters were responsible for this separation. The first canonical variate groups the three *P. depressa* samples (Fig 5.14), whilst the second canonical variate acts to separate the Tangiers sample from the other two *P. depressa* samples and the El Arenal sample from the other *P. caerulea* samples. The *P. caerulea* samples are grouped by canonical variate 1 and with the exception of the El Arenal sample the *P. caerulea* samples are grouped by canonical variate 2. The plots of canonical variate 2 against canonical variate 3 (Fig 5.15) and canonical variate 3 against canonical variate 4 (Fig 5.16) show little separation of the two species, thus it is clear that it is the first canonical vari-

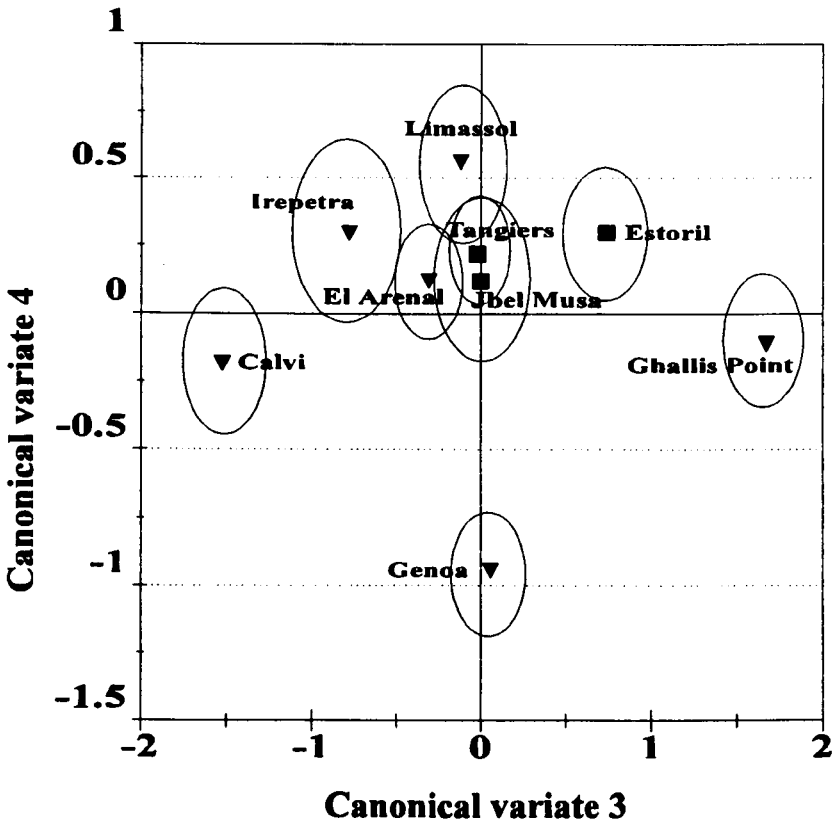


Figure 5.16 - Plot of canonical variate 3 against canonical variate 4 for the three *P.depressa* (■)samples and the six *P.caerulea* (▽) samples. The data have been log10(x+1) transformed and standardized. The data are separated by site, with group centroids and 95% confidence intervals of the mean shown.

ate that differentiates the *P. depressa* samples from the *P. caerulea* samples. It is the second canonical variate that separates the Tangiers sample from the other two *P. depressa* samples.

Table 5.9 - Linear discriminant function analysis using cross-validation, showing the percentage of individuals from the three *P.depressa* samples and the six *P.caerulea* samples correctly assigned to their original groups (bold). The data are differentiated by site.

Site	*P.depressa* Estoril	*P.depressa* Tangiers	*P.depressa* Jbel Musa	*P.caerulea* El Arenal	*P.caerulea* Calvi	*P.caerulea* Genoa	*P.caerulea* Ghallis Point	*P.caerulea* Irepetra	*P.caerulea* Limassol	Total %
P.depressa **Estoril**	**57.00**	15.00	14.00	5.00	0.00	9.00	0.00	0.00	0.00	100
P.depressa **Tangiers**	8.48	**75.76**	7.88	1.21	0.61	4.85	0.61	0.61	0.00	100
P.depressa **Jbel Musa**	24.24	9.09	**43.94**	10.61	1.52	7.58	0.00	3.03	0.00	100
P.caerulea **El Arenal**	3.27	0.65	7.84	**56.86**	5.23	3.27	5.88	8.50	8.50	100
P.caerulea **Calvi**	1.04	1.04	1.04	1.04	**60.42**	9.38	1.04	13.54	11.46	100
P.caerulea **Genoa**	2.56	5.13	7.69	12.82	4.27	**58.97**	0.85	5.13	2.56	100
P.caerulea **Ghallis Point**	0.00	0.00	0.00	2.00	0.00	1.00	**77.00**	4.00	16.00	100
P.caerulea **Irepetra**	0.00	0.00	3.64	16.36	20.00	3.64	7.27	**34.55**	14.55	100
P.caerulea **Limassol**	0.00	1.25	0.00	3.75	13.75	2.50	11.25	12.50	**55.00**	100
% allocated to group	10.30	16.63	8.58	14.06	10.19	11.80	10.94	7.30	10.19	100
% of data set	10.73	17.70	7.08	16.41	10.30	12.55	10.73	5.90	8.58	100

The first canonical variate has no strongly dominant element (Table 5.6). It shows a negative correlation between shell weight and apex to anterior distance, with a positive contribution from the shell height (lengthways). There is also a negative width across the apex element and a positive shell length element. The three *P. depressa* samples have high group means along this axis compared with the *P. caerulea* samples. Thus relative to *P. caerulea* of a similar size *P. depressa* individuals tended to have a heavier shell, a more anterial apex position, a greater shell height and be narrower across the shell apex. The second canonical variate which differentiated the Tangiers sample from the Estoril and Jbel Musa samples accounts for 19.9% of the variance (Table 5.6). This variate is mainly a contrast between the two shell height measures giving an indication of the amount of curvature in the basal plane of the shell, individuals with a high score in this variate showing greater curvature in the basal plane. Also contributing to this variate are a positive anterior to apex distance and a negative maximum shell width measure. Figure 5.15 shows that the mean of the Tangiers sample is lower than that of the samples from Estoril and Jbel Musa. Individuals from the Tangiers sample showed less curvature in the basal plane of the shell, an apex closer to the anterior of the shell and a smaller maximum shell width than those from the other two *P. depressa* samples.

The results of the cross-validated linear discriminant function analysis are given in Table 5.9. The percentage of individuals correctly assigned to their group ranges from a minimum of 35% for Irepetra to a maximum of 77% for Ghallis Point. There are two major points of note from this analysis:

1) The percentage of individuals assigned to the correct group is lower in this analysis than where the data set was divided into the two species.

2) The majority of individuals incorrectly assigned are put in one of the other groups of the same species

5.3.5 - Inclusion of radula length in the morphometric analysis.

Where reference is made to the 'first analysis' in this section, it refers to the multivariate analysis of the three *P. depressa* samples and the six *P. caerulea* samples with the seven shell parameters used and radula length excluded. When the Jbel Musa site was excluded

from the analysis and the radula length included as a parameter, it is the radula length measure that, of all the parameters measured, showed the lowest correlation with other measures (Table 5.10). It is, therefore, the radula length measure that shows the most variation in its relationship to the other shell measures.

Table 5.10 - Correlation coefficients for data comparing the two *P. depressa* samples and the six *P. caerulea* samples. Data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize variance. Radula length is included in the analysis.

	Length	Width (max)	Height (width)	Heightl (length)	Apex	Width (apex)	Radula length	Cube root of weigth
Length	1.000							
Width (max)	0.948	1.000						
Height (width)	0.798	0.800	1.000					
Height (length)	0.813	0.792	0.979	1.000				
Apex	0.818	0.789	0.652	0.692	1.000			
Width (apex)	0.934	0.965	0.803	0.799	0.829	1.000		
Radula length	0.538	0.560	0.715	0.692	0.209	0.515	1.000	
Cube root of weight	0.911	0.898	0.909	0.914	0.708	0.886	0.721	1.000

Table 5.11 - Eigenvalues for data comparing the two *P. depressa* samples and the six *P. caerulea* samples. Data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize variance. Radula length is included in the analysis.

	Eigenvalue	Percentage of variance	Cumulative percentage
Component 1	6.465	80.8	80.8
Component 2	0.931	11.6	92.5
Component 3	0.312	3.9	96.3
Component 4	0.124	1.5	97.9
Component 5	0.080	1.0	98.9
Component 6	0.043	0.5	99.4
Component 7	0.029	0.4	99.8
Component 8	0.016	0.1	100.0

In the principal component analysis, the first principal component explains 80.8% of the variance which is lower than in the first analysis (Table 5.11). It can be seen from the eigenvector of principal component 1 (Table 5.12) that radula length contributes less to this component than do the other parameters. The eigenvector therefore deviates from that predicted for an isometric size vector (Jolicoeur, 1963). This is directly related to the low correlation coefficients for radula length. The reasons for this are covered in the discussion, it is sufficient to say at this point that the first principal component can still be treated as an approximation to an isometric size vector, as the extent of the deviation from the predicted value is not too great. This contention is backed up by the fact that there is no significant variations in the distributions of the sample site means principal component 1 (Fig 5.17) when compared with the first analysis. The same is true of the distributions of the individuals of the two species along the first principal component (Fig 5.18).

Table 5.12 - Eigenvectors for data comparing the two *P. depressa* samples and the six *P. caerulea* samples. Data have been log10(x+1) transformed and a correlation matrix used to standardize variance. $p^{-1/2}$ is the predicted score for each element in principal component 1 if principal component 1 is an isometric size vector. Radula length is included in the analysis.

	Principal component 1	Principal component 2	Principal component 3	Principal component 4
% of variance	80.6	11.6	3.9	1.5
Length	0.373	-0.186	0.724	-0.779
Width (max)	0.372	0.163	0.403	-0.227
Height (width)	0.365	0.226	-0.469	-0.281
Height (length)	0.366	0.182	-0.534	-0.121
Apex	0.315	-0.537	-0.283	0.711
Width (apex)	0.371	-0.220	0.278	-0.175
Radula length	0.266	0.712	0.313	0.547
Cube root of weight	0.381	0.112	0.045	-0.116
$p^{-1/2}$	0.353			

In the first analysis , coupled with isometric size (principal component 1), it was principal component 2 that separated *P. depressa* from *P. caerulea*, this also holds true in this analysis. Indeed, from the distributions along principal component 2 (Fig 5.19), the separation of *P. depressa* from *P. caerulea* is stronger than in the first analysis ($F=1484.87$, $p>0.001$). The separation of the two species along this axis is clearly reflected in the distri-

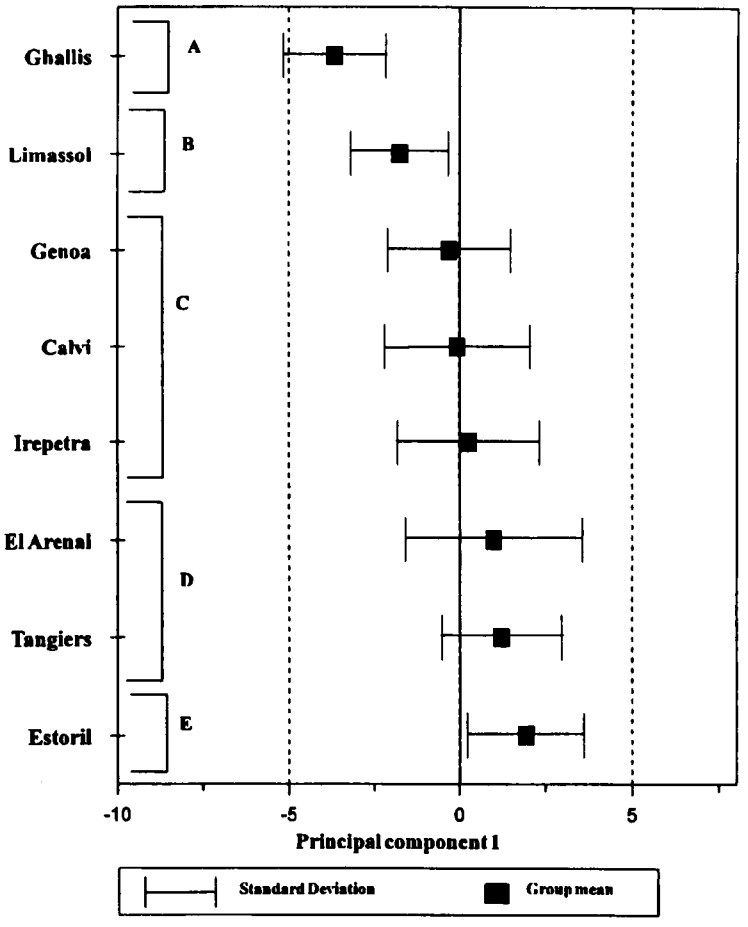


Figure 5.17 - Distribution of the group means of the two *P.depressa* samples and the six *P.caerulea* samples along principal component 1, with standard deviations shown. There are significant differences between groups ($F=89.62$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. Radula length is included in the analysis.

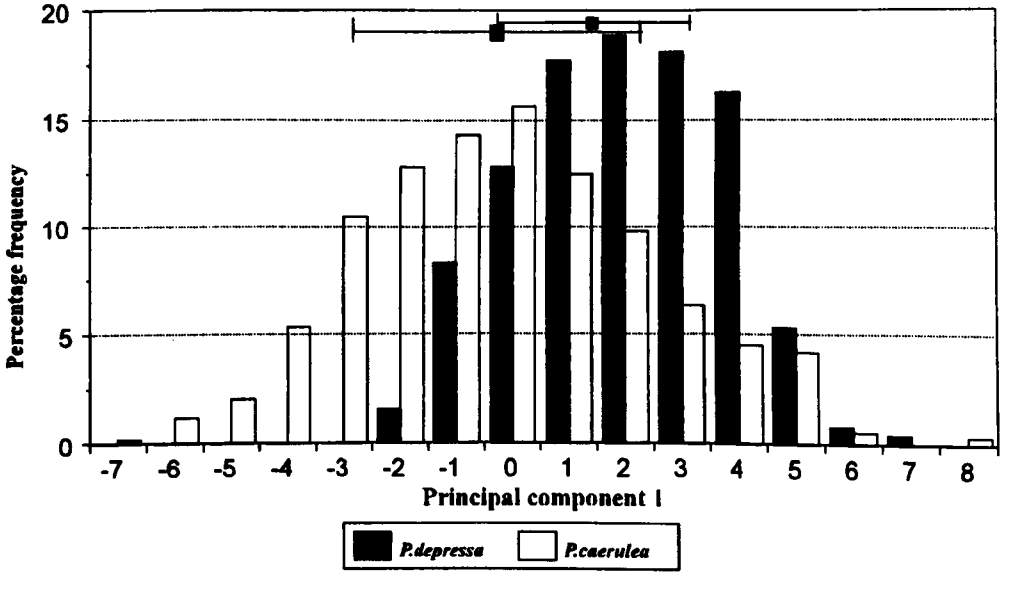


Figure 5.18 - Percentage distribution along principal component 1 for *P.depressa* and *P.caerulea* for combined samples. The data have been $\log_{10}(x+1)$ transformed and standardized. The data are separated by species, with group mean and 95% condfidence interval of the population shown. There are significant differences between groups ($F=150.39$, $p<0.001$). Radula length is included in the analysis.

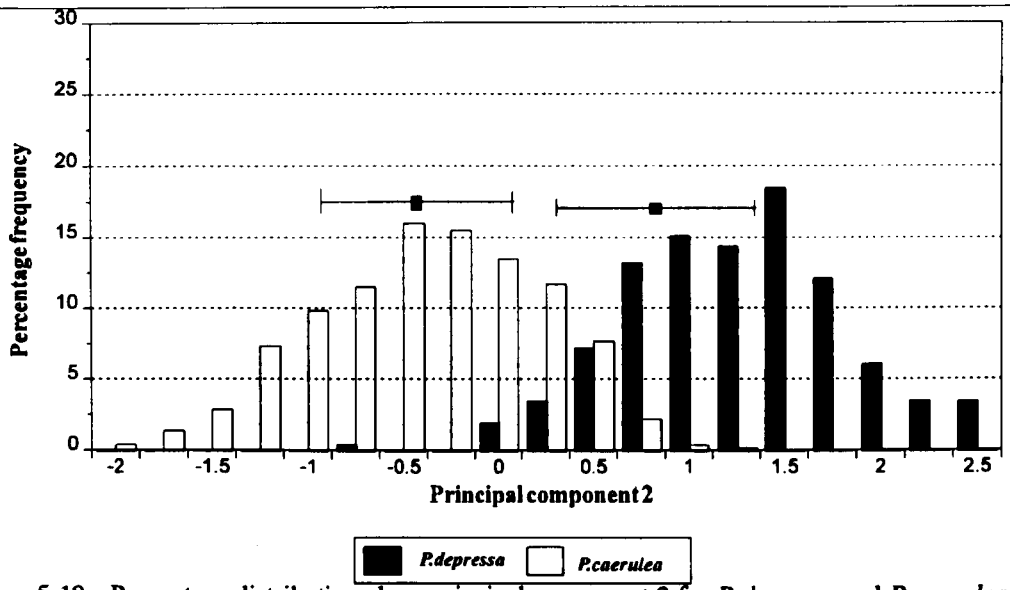


Figure 5.19 - Percentage distribution along principal component 2 for *P.depressa* and *P.caerulea* for combined samples. The data are separated by species, with group mean and 95% condfidence interval of the population shown. There are significant differences between groups ($F = 1484.87$, $p < 0.001$). Radula length is included in the analysis.

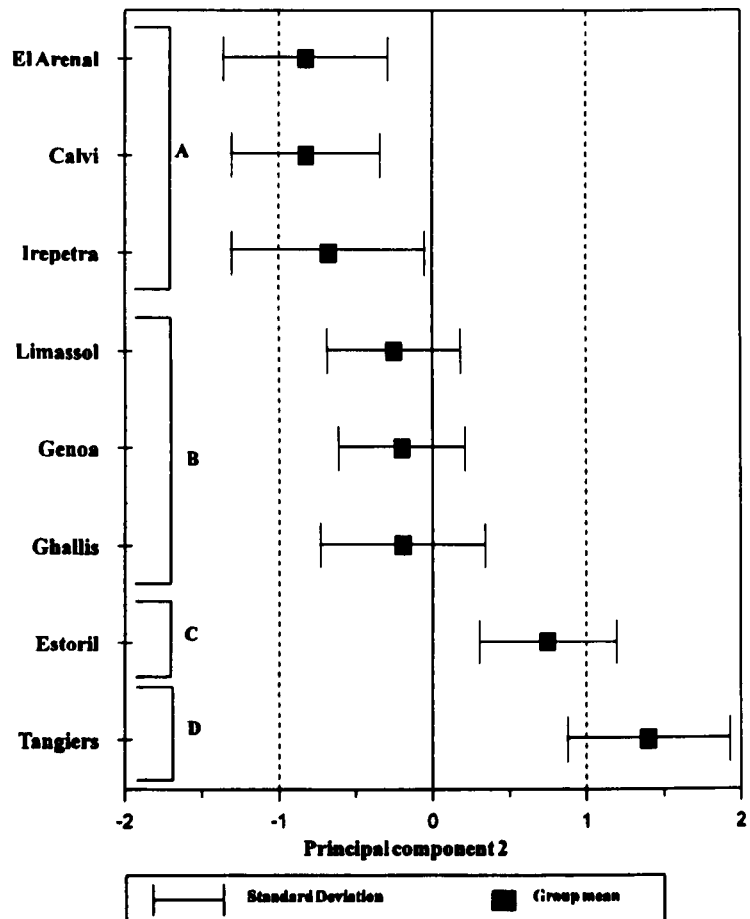


Figure 5.20 - Distribution of the group means of the two *P.depressa* samples and the six *P.caerulea* samples along principal component 2, with standard deviations shown. There are significant differences between groups ($F = 331.76$, $p < 0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data. Radula length is included in the analysis.

bution of eight sample site group means (Fig 5.20). The Tangiers and Estoril sites have the two highest values and are significantly different from all six of the *P. caerulea* samples. Interestingly the Tangiers sample is significantly different from the Estoril sample along this axis. The second principal component, which accounts for 11.6% of the variance (Table 5.12) is dominated by a contrast between radula length and apex to anterior distance. The *P. depressa* samples, which have high group means for this component, will have a longer radula and apex nearer to the anterior of the shell, relative to *P. caerulea* of similar size. The separation of *P. depressa* and *P. caerulea* along the axes of principal component 1 and principal component 2 can be seen clearly in a bivariate plot of the individuals of the two species (Fig 5.21) and of the eight sample group means (Fig 5.22).

Table 5.13 - Standardized canonical coefficients for the *P.depressa* samples and the *P.caerulea* samples differentiated by species. The data have been log10(x+1) transformed and standardized. Radula length is included in the analysis.

Measured character	Canonical variate 1
Length	-0.540
Width (max)	0.364
Height (width)	-0.054
Height (length)	0.327
Apex	-0.441
Width (apex)	-0.836
Radula length	2.125
Cube root of weight	0.611

Although there are significant differences between the group means for the two species along principal component 3 ($F=7.99$, $p<0.01$) and principal component 4 ($F=17.03$, $p<0.001$) there is a great degree of overlap in these distributions. This can be seen in the distributions of the eight sample group means along principal component 3 and 4, where there is no species specific differentiation (Fig 5.23 and 5.24).

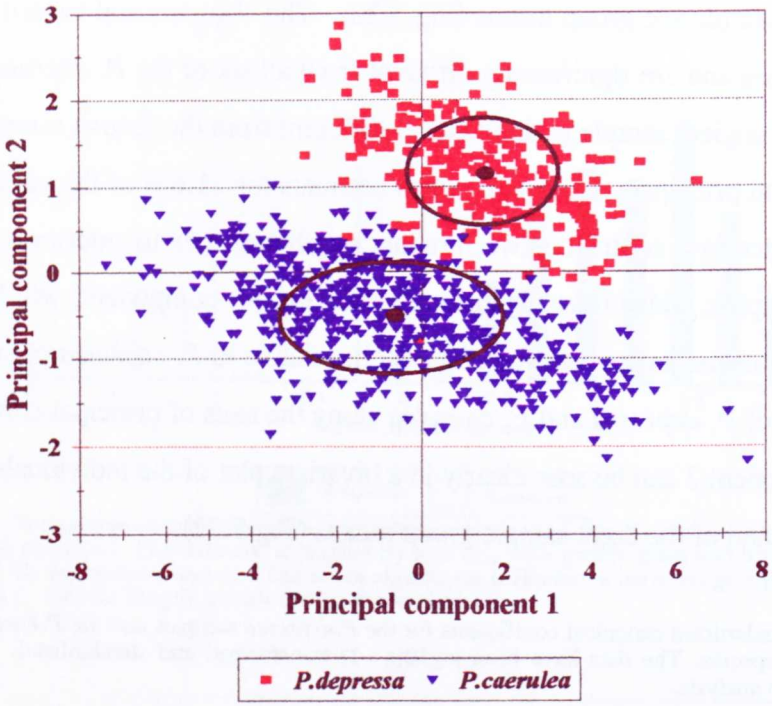


Figure 5.21 - Plot of principal component 1 against principal component 2 for the two *P.depressa* samples and the six *P.caerulea* samples. The data are differentiated by species. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data. The data are separated by species, with group centroids and standard deviations shown. Radula length is included in the analysis.

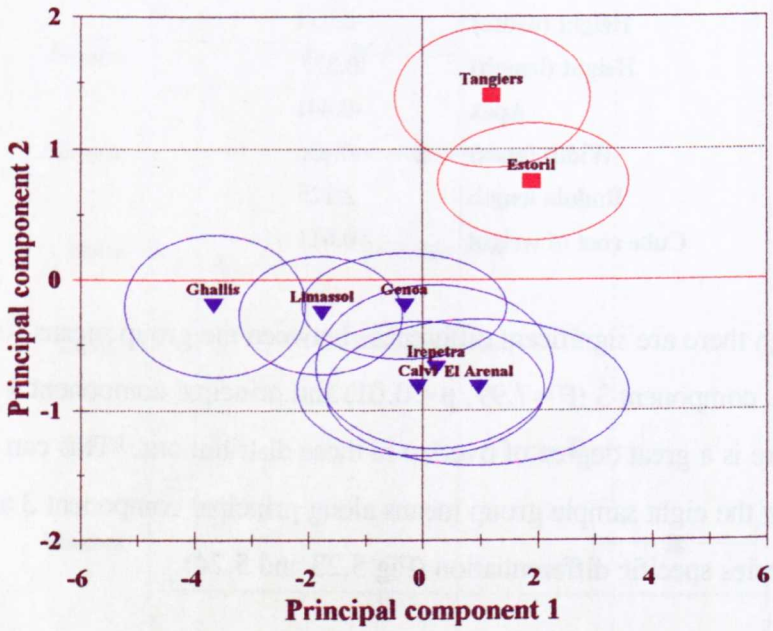


Figure 5.9 - Plot of principal component 1 against principal component 2 for the two *P.depressa* samples and the six *P.caerulea* samples. The data are differentiated by species. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data. The data are separated by site, with group centroids and standard deviations shown.

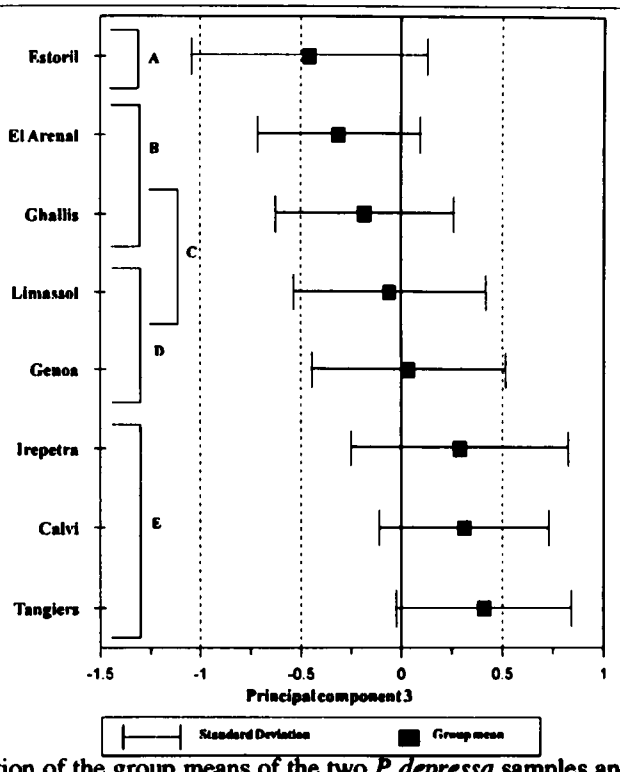


Figure 5.23 - Distribution of the group means of the two *P.depressa* samples and the six *P.caerulea* samples along principal component 3, with standard deviations shown. There are significant differences between groups ($F=52.73$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data. Radula length is included in the analysis.

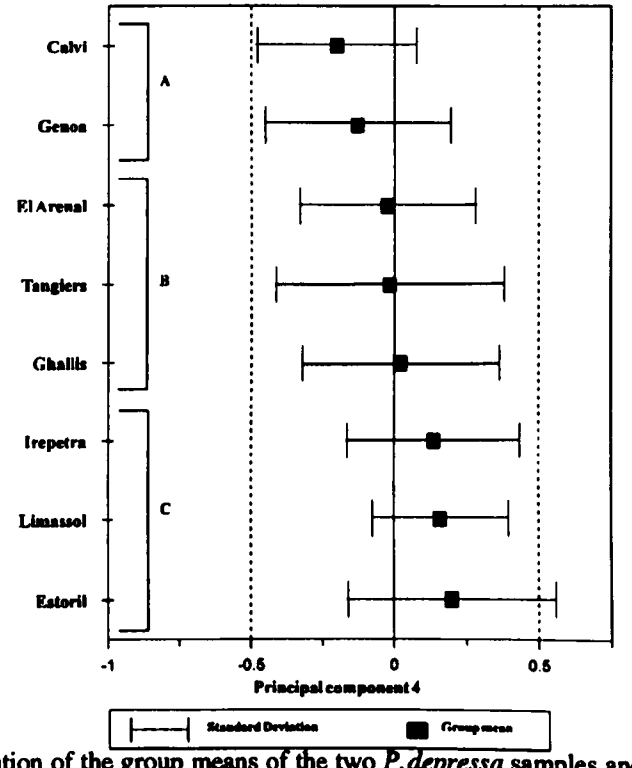


Figure 5.24 - Distribution of the group means of the two *P.depressa* samples and the six *P.caerulea* samples along principal component 2, with standard deviations shown. There are significant differences between groups ($F=17.03$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data. Radula length is included in the analysis.

Table 5.14 - Linear discriminant function using cross-validation showing percentage of individuals from the *P. depressa* samples and the *P. caerulea* samples correctly assigned to their original groups. The data are differentiated by species. Radula length is included in the analysis.

Site	*P. depressa*	*P. caerulea*
P. depressa	**98.90**	1.13
P. caerulea	0.67	**99.32**
Percentage allocated to group	30.72	69.28
Percentage of original data set	30.60	69.40

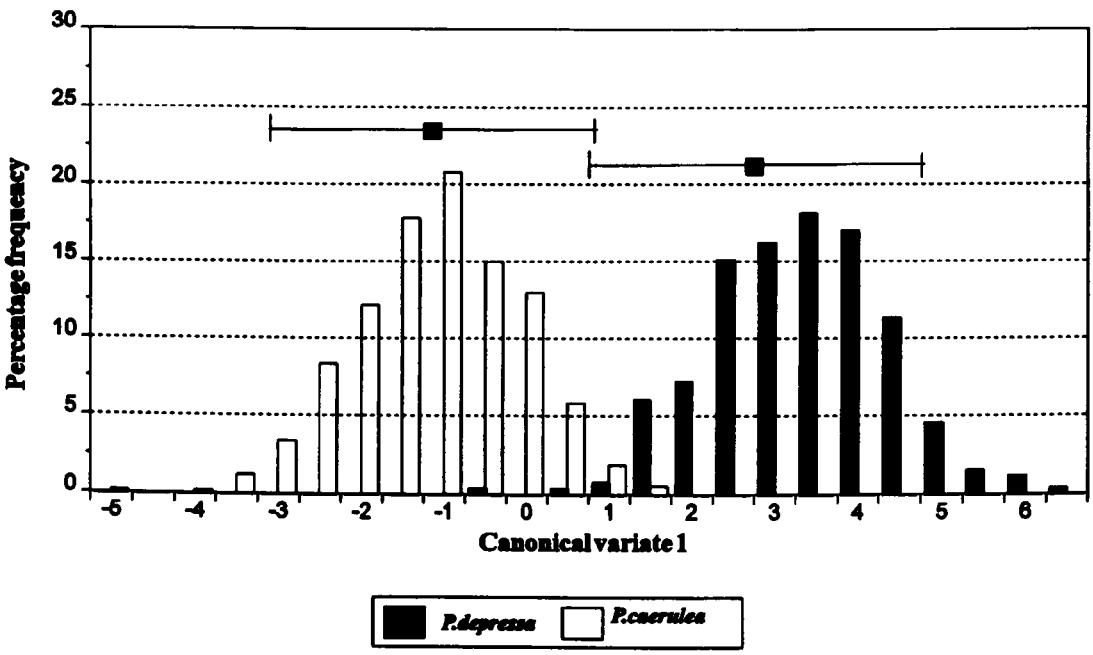


Figure 5.25 - Percentage distribution along canonical variate 1 for *P.depressa* and *P.caerulea* for combined samples. The data have been $\log_{10}(x+1)$ transformed and standardized. The data are separated by species, with group centroids and 95% confidence intervals shown. Radula length is included in the analysis and only eight sites are used.

Carrying out a canonical variate analysis on the data split into the two species the analysis shows a higher degree of separation of the species than that found in the first analysis. The frequency distribution of individuals of the two species along the single canonical variate generated, show almost complete separation of *P. depressa* from *P. caerulea* (Fig 5.25), with the 95% confidence intervals of the populations not overlapping. The major coefficients influencing this separation are radula length and shell width across the apex. There is a negative correlation between these two coefficients (Table 5.13). The other coefficients influencing this separation are a positive shell weight and a negative shell length component.

Table 5.15 - Canonical pairwise squared distances between groups for the two *P.depressa* samples and the six *P.caerulea* samples differentiated by site. The data have been $\log_{10}(x+1)$ transformed and standardized. Radula length is included in the analysis.

Site	*P.depressa* Estoril	*P.depressa* Tangiers	*P.caerulea* El Arenal	*P.caerulea* Calvi	*P.caerulea* Genoa	*P.caerulea* Ghallis Point	*P.caerulea* Irepetra	*P.caerulea* Limassol
P.depressa **Estoril**	0							
P.depressa **Tangiers**	9.610	0						
P.caerulea **El Arenal**	20.950	45.141	0					
P.caerulea **Calvi**	26.184	40.880	6.956	0				
P.caerulea **Genoa**	12.675	28.127	5.195	5.694	0			
P.caerulea **Ghallis Point**	30.140	50.168	10.997	11.776	10.220	0		
P.caerulea **Irepetra**	19.985	33.440	6.249	3.378	6.810	8.510		
P.caerulea **Limassol**	20.606	35.553	8.057	4.983	8.073	5.674	1.879	0

Thus this indicates (from Fig 5.25) that *P. depressa* has a longer radula , a shell narrower across the apex and a greater shell weight than *P. caerulea*. A cross-validated linear discriminant function analysis shows a higher level of correct assignment of the two species than that found in the first analysis, with approximately 99% of individuals being correctly assigned in both species (Table 5.14). In the canonical variate analysis based on the eight sample sites, when compared with the results of the first analysis, the pairwise squared distances between sites have increased slightly in within-species comparisons (Table 5.15). The distances in canonical space between the two *P. depressa* sites and the six *P. caerulea* sites have increased markedly over those from the first analysis.

Table 5.16 - Group means of the first five canonical variates for the two *P. depressa* samples and the six *P. caerulea* samples differentiated by site. The data have been $\log_{10}(x+1)$ transformed and standardized. Radula length is included in the analysis.

SITE	Canonical variate 1	Canonical variate 2	Canonical variate 3	Canonical variate 4	Canonical variate 5
P. depressa Estoril	2.333	1.111	0.928	0.404	-0.671
P. depressa Tangiers	4.423	-0.501	-0.259	-0.072	0.312
P. caerulea El Arenal	-2.002	1.387	0.023	0.382	0.432
P. caerulea Calvi	-1.773	-0.338	-1.638	-0.319	-0.375
P. caerulea Genoa	-0.626	0.598	-0.015	-1.143	-0.114
P. caerulea Ghallis Point	-2.360	-1.435	1.515	-0.375	0.148
P. caerulea Irepetra	-1.232	-0.711	-0.714	0.798	0.253
P. caerulea Limassol	-1.369	-1.192	-0.084	0.889	-0.374

Looking at a bivariate plot of canonical variate 1 against canonical variate 2 (Fig 5.26) with the group means for the eight sites (From Table 5.16) and the 95% confidence limits of the means shown, the separation of the two species is very evident. Indeed, with the 95% confidence limit of the population for any group being 1.9, the group mean for any site does not fall within the 95% confidence region of any sample of the other species. It is canonical variate 1 that acts to differentiate between the two species, whilst canonical variate 2 separates the Estoril sample from the Tangiers sample of *P. depressa*. The plot of canonical variate 3 against canonical variate 4 shows no species specific separation along either axis (Fig 5.27).

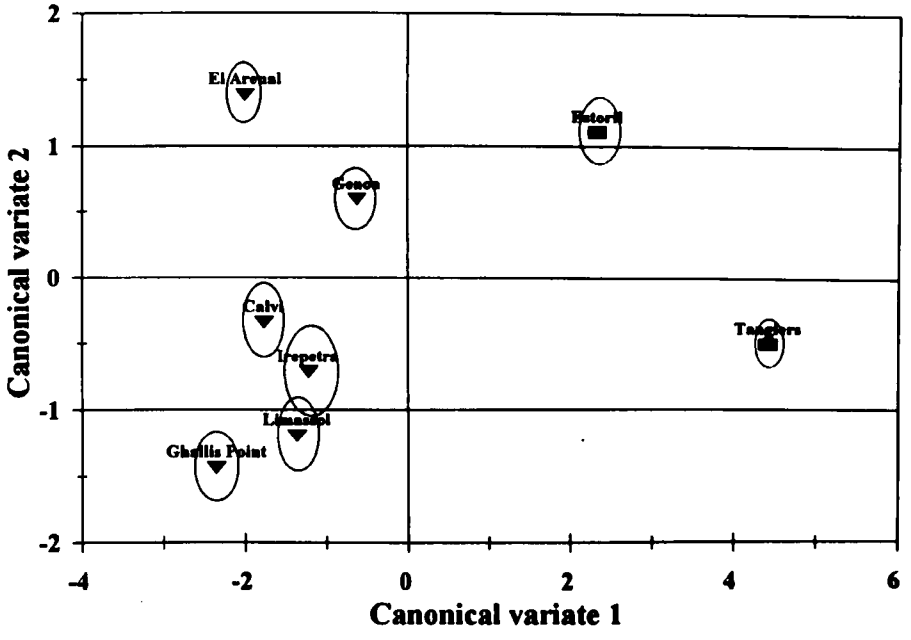


Figure 5.26 - Plot of canonical variate 1 against canonical variate 2 for the two *P.depressa* (■)samples and the six *P.caerulea* (▽) samples. The data are separated by site, with group centroids and 95% confidence intervals of the mean shown. Radula length included in the analysis.

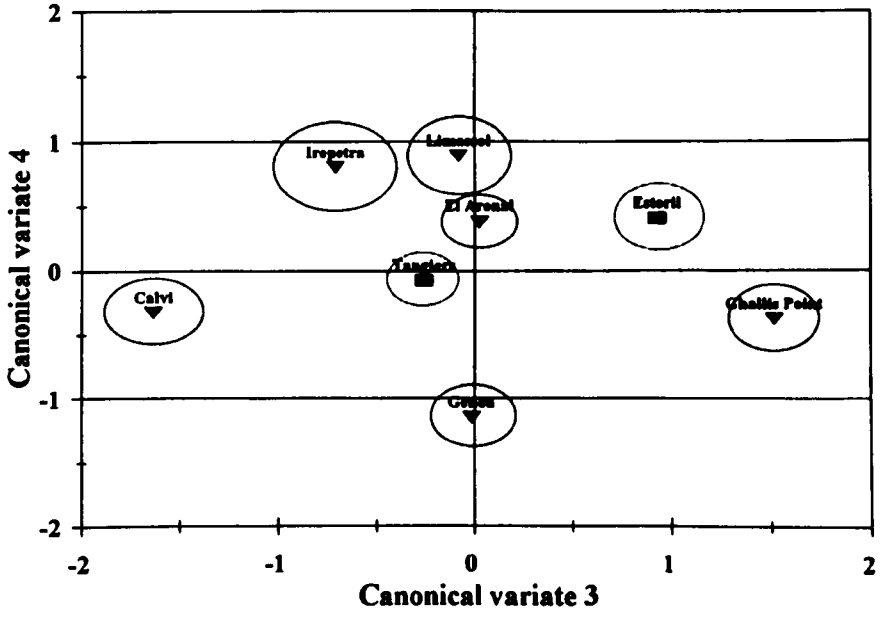


Figure 5.27 - Plot of canonical variate 2 against canonical variate 3 for the two *P.depressa* (■)samples and the six *P.caerulea* (▽) samples. The data are separated by site, with group centroids and 95% confidence intervals of the mean shown. Radula length included in the analysis.

Table 5.17 - Standardized canonical coefficients for the two *P. depressa* samples and the six *P. caerulea* samples differentiated by site. The data have been $\log_{10}(x+1)$ transformed and standardized. Radula length is included in the analysis.

Measured character	Canonical variate 1	Canonical variate 2	Canonical variate 3	Canonical variate 4	Canonical variate 5
Percentage of variance	73.4	11.5	8.2	4.2	1.6
Length	0.054	1.394	-2.695	-0.911	-0.519
Width (max)	0.468	-0.478	-1.232	1.203	-0.139
Height (width)	0.158	-2.355	-2.370	0.201	-4.153
Height (length)	-0.203	3.439	2.482	1.000	4.220
Apex	-1.029	-0.100	0.693	1.427	-1.459
Width (apex)	-0.775	-1.053	0.693	0.061	2.974
Radula length	2.499	-0.793	0.122	1.165	-0.029
Cube root of weight	0.537	0.637	1.925	-3.162	-1.074

So, as in the first analysis, it is the first canonical variate that separates *P. depressa* sites from *P. caerulea* sites and the second canonical variate that separates the Tangiers from the Estoril sample. The first canonical variate accounts for 73.4% of the variance (Table 5.17). This variate is dominated by radula length and apex to anterior distance, with the two elements negatively correlated. A negative width across apex and a positive shell weight element also contribute significantly. As in the first analysis canonical variate 2 is a contrast of the two height measures, indicating curvature in the basal plane of the shell. These results indicate that *P. depressa* has a longer radula, apex nearer the anterior of the shell, a greater shell weight and a narrower shell across the apex than *P. caerulea*.

The cross-validated linear discriminant function analysis of the eight sites shows in all cases a higher proportion of individuals assigned correctly, than in the first analysis (Table 5.18). The higher assignment within the *P. caerulea* sites is due to the increased powers of discrimination due to the use of radula length in the analysis. The higher correct assignment in the two *P. depressa* samples is due partly to the inclusion of radula length and partly due to the fewer number of *P. depressa* sites included. As the majority of incorrect assignments are placed in a sample of the same species and the differences between the two species are quite marked, the removal of a sample of a species will increase the proportion of individuals correctly assigned within that species.

Table 5.18 - Linear discriminant function using cross-validation, showing percentage of individuals from the two *P.depressa* samples and the six *P.caerulea* samples correctly assigned to their original groups (bold). The data are differentiated by site. Radula length is included in the analysis.

Site	*P.depressa* Estoril	*P.depressa* Tangiers	*P.caerulea* El Arenal	*P.caerulea* Calvi	*P.caerulea* Genoa	*P.caerulea* Ghallis Point	*P.caerulea* Irepetra	*P.caerulea* Limassol
P.depressa **Estoril**	**87.00**	11.00	1.00	0.00	1.00	0.00	0.00	0.00
P.depressa **Tangiers**	6.67	**93.73**	0.00	0.00	0.00	0.00	0.61	0.00
P.caerulea **El Arenal**	0.65	0.00	**69.28**	7.84	5.23	8.50	5.23	3.27
P.caerulea **Calvi**	1.04	0.00	3.12	**67.71**	5.21	3.12	12.50	7.29
P.caerulea **Genoa**	2.56	0.00	11.11	5.98	**73.50**	1.71	2.56	2.56
P.caerulea **Ghallis Point**	0.00	0.00	2.00	0.00	1.00	**83.00**	4.00	10.00
P.caerulea **Irepetra**	0.00	0.00	7.27	20.00	5.45	1.82	**43.64**	21.82
P.caerulea **Limassol**	0.00	0.00	2.50	8.75	1.25	12.00	16.25	**58.75**
% allocatedto group	11.89	18.94	15.13	11.78	12.12	12.93	7.51	9.70
% of data set	11.55	19.05	17.67	11.09	13.51	11.55	6.35	9.24

5.3.6 - Electrophoretic analysis.

Of the thirteen loci assayed five were polymorphic in both *P. depressa* and *P. caerulea*: *Pgi*, *Pgm*, *G3pdh*, *Pgdh* and *Np* (Table 5.19). Of these five loci *Pgi* and *Np* had a different most common allele for the two species. At the *Pgi* locus, allele D was most common in *P. depressa* whilst for *P. caerulea* allele B was. At the *Np* locus allele D was most common in *P. depressa* and allele B in *P. caerulea*. *Mpi* was fixed for allele D in *P. depressa* and generally polymorphic in *P. caerulea*. *Mdh-1* and *Idh-2* were fixed for different alleles in the two species. *Mdh-2*, *Idh-2*, *Me* (not polymorphic by the 95% criterion) and *Got* were fixed for the same allele in all samples. The *Ald-1* locus was fixed in *P. caerulea*, and fixed for a different allele in two of the three *P. depressa* samples (the Estoril sample of *P. depressa* was polymorphic).

Table 5.20 - Summary of the genetic variability in the three *P. depressa* samples and the six *P. caerulea* samples. Shown are the mean sample size per locus; the mean number of alleles per locus; percentage of polymorphic loci (Commonest allele does not exceed 95%) and the mean observed and expected (Nei's unbiased estimate, 1978) heterozygosity proportions.

Population	Mean Sample size per locus	Mean No. of alleles per locus	Percentage of loci polymorphic	Mean Heteozygosity Observed	Mean Heteozygosity H-W expected
***P. depressa*, Estoril**	80.8	1.5	23.1	0.051	0.066
S.E.	± 2.5	± 0.2		± 0.023	± 0.033
***P. depressa*, Tangiers**	82.2	1.5	30.8	0.05	0.079
S.E.	± 4.2	± 0.2		± 0.025	± 0.036
***P. depressa*, Jbel Musa**	63.3	1.5	38.5	0.085	0.120
S.E.	± 0.6	± 0.2		± 0.032	± 0.044
***P. caerulea*, El Arenal**	100.8	1.7	46.2	0.104	0.131
S.E.	±1.8	±0.2		±0.037	±0.046
***P. caerulea*, Calvi**	92.8	1.9	46.2	0.099	0.122
S.E.	±2.3	±0.3		±0.033	±0.042
***P. caerulea*, Genoa**	40.0	1.8	38.5	0.122	0.128
S.E.	±1.5	±0.3		±0.064	±0.053
***P. caerulea*, Ghallis Point**	72.7	1.5	30.8	0.075	0.107
S.E.	±2.8	±0.2		±0.037	±0.053
***P. caerulea*, Irepetra**	52.7	1.8	46.2	0.087	0.215
S.E.	±1.1	±0.3		±0.035	±0.077
***P. caerulea*, Limassol**	76.8	1.9	46.2	0.115	0.206
S.E.	±2.2	±0.3		±0.049	±0.071

Table 5.19 - Allele frequencies and sample size (N) at thirteen loci for the three *P.depressa* and the six *P.caerulea* samples. A key to the sites is given at the end of the table.

SITE	1	2	3	4	5	6	7	8	9
Locus									
Pgi-1									
(N)	**76**	68	**65**	**113**	**97**	**70**	**78**	**55**	**79**
A	0.000	0.000	0.000	0.000	0.113	0.164	0.173	0.364	0.316
B	0.000	0.000	0.000	0.836	0.876	0.829	0.827	0.600	0.658
C	0.026	0.029	0.031	0.164	0.000	0.000	0.000	0.036	0.025
D	0.895	0.809	0.846	0.000	0.010	0.007	0.000	0.000	0.000
E	0.079	0.162	0.123	0.000	0.000	0.000	0.000	0.000	0.000
Pgm-1									
(N)	**87**	76	**66**	**108**	**93**	**73**	**75**	**50**	**66**
A	0.000	0.000	0.000	0.000	0.054	0.527	0.487	0.280	0.462
B	0.000	0.000	0.000	0.028	0.758	0.178	0.207	0.180	0.242
C	0.029	0.026	0.159	0.213	0.124	0.027	0.027	0.220	0.106
D	0.971	0.974	0.841	0.759	0.065	0.267	0.280	0.320	0.189
G3pdh									
(N)	**85**	88	**63**	**111**	**90**	**79**	**61**	**45**	**54**
A	0.965	0.114	0.310	0.005	0.156	0.930	0.959	0.244	0.667
B	0.035	0.886	0.690	0.167	0.800	0.070	0.041	0.756	0.333
C	0.000	0.000	0.000	0.829	0.044	0.000	0.000	0.000	0.000
Pgdh									
(N)	**61**	55	**60**	**107**	**70**	**70**	**55**	**46**	**80**
A	0.918	0.945	0.783	0.687	0.900	0.893	0.836	0.565	0.619
B	0.082	0.000	0.000	0.299	0.086	0.086	0.164	0.272	0.231
C	0.000	0.055	0.217	0.014	0.014	0.021	0.000	0.163	0.150
D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Np									
(N)	**80**	54	**63**	**99**	**97**	**69**	**80**	**55**	**79**
A	0.000	0.000	0.000	0.086	0.088	0.043	0.050	0.064	0.082
B	0.000	0.222	0.119	0.914	0.912	0.957	0.950	0.936	0.905
C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
D	1.000	0.778	0.881	0.000	0.000	0.000	0.000	0.000	0.013
Mpi-1									
(N)	**80**	80	**66**	**94**	**80**	**82**	**50**	**49**	**80**
A	0.000	0.000	0.000	0.090	0.081	0.049	1.000	0.337	0.225
B	0.000	0.000	0.000	0.910	0.844	0.793	0.000	0.000	0.000
C	0.000	0.000	0.000	0.000	0.075	0.159	0.000	0.663	0.769
D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
E	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000
Mdh-1									
(N)	**80**	80	**63**	**97**	**97**	**82**	**78**	**55**	**80**
A	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000
B	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000
Mdh-2									
(N)	**80**	95	**64**	**97**	**97**	**82**	**78**	**55**	**80**
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Ald-1									
(N)	**98**	98	**66**	**97**	**97**	**82**	**78**	**55**	**80**
A	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.735	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000
C	0.265	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 5.19 (continued) - Allele frequencies and sample size (N) at thirteen loci for the three *P.depressa* and the six *P.caerulea* samples. A key to the sites is given at the end of the table.

SITE	1	2	3	4	5	6	7	8	9
Locus									
Idh-1									
(N)	**76**	96	**60**	**97**	**97**	**82**	**78**	**55**	**80**
A	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000
B	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000
Idh-2									
(N)	**75**	98	**62**	**97**	**97**	**82**	**78**	**55**	**80**
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Me-2									
(N)	**80**	95	**64**	**97**	**97**	**82**	**78**	**55**	**80**
A	1.000	1.000	0.984	1.000	1.000	1.000	1.000	1.000	1.000
	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000
Got									
(N)	**80**	93	**64**	**97**	**97**	**82**	**78**	**55**	**80**
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Key to Sites

1 - *P.depressa*, Estoril, Portugal

2 - *P.depressa*, Tangiers, Morocco

3 - *P.depressa*, Jbel Musa, Morocco

4 - *P.caerulea*, El Arenal, Mallorca

5 - *P.caerulea*, Calvi, Corsica

6 - *P.caerulea*, Genoa, Italy

7 - *P.caerulea*, Ghallis Point, Malta

8 - *P.caerulea*, Irepetra, Crete

9 - *P.caerulea*, Limassol, Cyprus

Table 5.21 - Summary statistic of heterozygosity levels for the polymorphic loci in the three *P.depressa* samples and the six *P.caerulea* samples. Expected heterozygosite is calculated using Nei's unbiased estimate of heterozygosity. The loci fixation index (F) and disequilibrium coefficient (D) are given and the results of a $\chi 2$ test for significant departure from expected heterozygosity level.

Locus	Observed heterozygotes	Expected heterozygotes	Fixation index (F)	D	$\chi 2$	D.F.	*P*	Pooled
P.depressa **Estoril**								
Pgi-1	14	14.73	0.043	-0.049	0.065	1	NS	√
Pgm-1	5	4.88	-0.030	0.024	0.061	1	NS	
G3pdh	4	5.82	0.309	-0.313	9.936	1	**	
Pgdh	8	9.26	0.129	-0.136	1.239	1	NS	
Ald-1	24	38.40	0.372	-0.375	14.005	1	***	
P.depressa **Tangiers**								
Pgi-1	16	21.84	0.262	-0.267	8.089	1	*	√
Pgm-1	4	3.92	-0.027	0.020	0.041	1	NS	
G3pdh	12	17.83	0.323	-0.327	9.851	1	**	
Pgdh	0	5.73	1.000	-1.000	65.429	1	***	
Np	12	18.84	0.357	-0.363	7.379	1	**	
P.depressa **Jbel Musa**								
Pgi-1	13	17.55	0.254	-0.259	5.971	1	*	
Pgm-1	13	17.79	0.264	-0.269	4.999	1	*	√
G3pdh	21	27.14	0.220	-0.226	3.298	1	NS	
Pgdh	12	20.54	0.411	-0.416	10.718	1	**	
Np	9	13.32	0.319	-0.324	7.051	1	**	
Me-2	2	1.98	-0.016	0.008	0.008	1	NS	
P.caerulea **El Arenal**								
Pgi-1	25	31.08	0.192	-0.196	4.43	1	*	
Pgm-1	32	40.95	0.215	-0.219	9.49	1	**	√
G3pdh	25	31.81	0.210	-0.214	6.62	1	*	√
Pgdh	39	47.14	0.169	-0.173	2.66	1	NS	√
Np	11	15.62	0.292	-0.296	9.16	1	**	
Mpi-1	13	15.55	0.159	-0.164	2.67	1	NS	
P.caerulea **Calvi**								
Pgi-1	20	21.37	0.059	-0.064	0.29	1	NS	√
Pgm-1	30	37.68	0.200	-0.204	2.26	1	NS	√
G3pdh	24	30.21	0.201	-0.206	2.71	1	NS	√
Pgdh	10	12.86	0.217	-0.223	3.37	1	NS	√
Np	15	15.59	0.033	-0.038	0.15	1	NS	
Mpi-1	16	22.21	0.275	-0.286	7.13	1	NS	√

Table 5.21 (continued) - Summary statistic of heterozygosity levels for the polymorphic loci in the three *P.depressa* samples and the six *P.caerulea* samples. Expected heterozygosite is calculated using Nei's unbiased estimate of heterozygosity. The loci fixation index (F) and disequilibrium coefficient (D) are given and the results of a $\chi 2$ test for significant departure from expected heterozygosity level.

Locus	Observed hetero-zygotes	Expected hetero-zygotes	Fixation index (F)	D	$\chi 2$	D.F.	*P*	Pooled
***P.caerulea* Genoa**								
Pgi-1	16	20.19	0.202	-0.208	2.94	1	NS	√
Pgm-1	61	45.43	-0.352	0.343	22.85	1	***	√
G3pdh	7	10.30	0.316	-0.320	8.87	1	**	
Pgdh	11	13.75	0.194	-0.200	2.54	1	NS	√
Np	4	5.78	0.303	-0.308	7.80	1	**	
Mpi-1	18	28.39	0.362	-0.366	14.13	1	***	√
***P.caerulea* Ghallis Point**								
Pgi-1	15	22.47	0.328	-0.332	8.91	1	**	
Pgm-1	33	48.42	0.314	-0.319	6.14	1	*	√
G3pdh	3	4.84	0.374	-0.379	10.89	1	**	
Pgdh	12	15.19	0.203	-0.210	2.55	1	NS	
Np	6	7.65	0.211	-0.215	4.22	1	*	
***P.caerulea* Irepetra**								
Pgi-1	10	28.11	0.641	-0.644	29.37	3	***	√
Pgm-1	19	37.29	0.485	-0.491	6.77	1	**	√
G3pdh	6	16.81	0.639	-0.643	19.35	1	***	
Pgdh	13	26.98	0.513	-0.518	14.89	1	***	√
Np	5	6.62	0.237	-0.244	3.79	1	NS	
Mpi-1	3	22.11	0.863	-0.864	37.56	1	***	
***P.caerulea* Limassol**								
Pgi-1	6	37.05	0.837	-0.838	63.30	1	***	√
Pgm-1	37	45.26	0.176	-0.182	0.23	1	NS	√
G3pdh	12	24.22	0.500	-0.505	14.08	1	***	
Pgdh	29	43.57	0.336	-0.334	4.54	1	*	√
Np	13	13.83	0.054	-0.060	0.20	1	NS	√
Mpi-1	9	28.85	0.686	-0.688	38.47	1	***	√

Table 5.22. - Nei's (1978) unbiased genetic identity over thirteen loci between the three *P.depressa* samples and the six *P.caerulea* samples.

Site	*P.depressa* Estoril	*P.depressa* Tangiers	*P.depressa* Jbel Musa	*P.caerulea* El Arenal	*P.caerulea* Calvi	*P.caerulea* Genoa	*P.caerulea* Ghallis Point	*P.caerulea* Irepetra	*P.caerulea* Limassol
P.depressa **Estoril**	X								
P.depressa **Tangiers**	0.930	X							
P.depressa **Jbel Musa**	0.953	0.993	X						
P.caerulea **El Arenal**	0.462	0.495	0.477	X					
P.caerulea **Calvi**	0.432	0.501	0.480	0.904	X				
P.caerulea **Genoa**	0.511	0.472	0.472	0.898	0.923	X			
P.caerulea **Ghallis Point**	0.504	0.460	0.460	0.831	0.855	0.932	X		
P.caerulea **Irepetra**	0.460	0.522	0.522	0.865	0.915	0.898	0.904	X	
P.caerulea **Limassol**	0.486	0.483	0.483	0.846	0.894	0.940	0.934	0.980	X

A number of loci can be regarded as diagnostic in distinguishing *P. depressa* and *P. caerulea* in this study. These are *Mpi*, *Mdh-1*, *Ald-1* and *Idh-1*. The presence of diagnostic loci is of huge benefit in identifying samples of limpets where the species cannot be unambiguously determined on morphological grounds.

The percentage of polymorphic loci was lower for the Estoril sample than any of the other samples (Table 5.20). Using Nei's (1978) estimate of expected heterozygosity all the samples show a deficit of heterozygotes. Looking at these deficiencies at locus level, where rare alleles were present that could bias the results, pooling of these rare alleles was carried out and this is indicated in the right hand column of the table.(Table 5.21). The Estoril sample shows significant heterozygote deficiencies at the *G3pdh* and *Ald-1* loci (p). Both the Tangiers and the Jbel Musa samples showed significant heterozygote deficiencies at the *Pgdh* and *Np* loci, additionally the Tangiers sample shows significant heterozygote deficiencies at the *G3pdh* locus. No sample showed heterozygote deficiencies at all loci.

Using Nei's genetic identity (1978) as a measure of relatedness, pairwise distances between samples were generated (Table 5.22). The two groups with the highest identity were the two samples of *P. depressa* from Morocco (Tangiers and Jbel Musa) with an identity of 0.993. The lowest identity between any two groups was 0.432 between *P. caerulea* from Calvi and *P. depressa* from Estoril. The pairwise distances were used in a UPGMA cluster analysis (Fig 5.28). The two species are very clearly separated with the common identity at about 0.50, which is consistent with the two species being con-generic. The two *P. depressa* samples from Morocco were more closely related to each other than to the *P. depressa* sample from Estoril. The common identity level between the samples of *P. depressa* is about 0.95 which is well within the 0.85 level given for con-specifics, the common identity of 0.89 for *P. caerulea* is also within this level (for more details of the genetic results of *P. caerulea* see chapter 4).

Analysis of heterogeneity of allele frequencies (Table 5.23) looks for significant differences in allele frequencies between samples from the same species. At the 1% level both of the two species show significant differences in allele frequencies between samples. At locus level *P. depressa* shows significant heterogeneity in five out of seven loci, whilst *P. caerulea* at five out of six.

Table 5.23 - Summary of Chi-squared statistics for the analysis of heterogeneity of allele frequencies over the polymorphic loci between the samples of the same species. Shown are Number of alleles, Chi-squared value, degrees of freedom (D.F.) and probability level (p), where N.S. = not significant.

P. depressa

Locus	No. of alleles	χ^2	D.F.	p
Pgi-1	3	4.815	4	NS
Pgm-1	2	26.544	2	***
G3pdh	2	269.610	2	***
Pgdh	3	54.428	4	***
Np	2	36.537	2	***
Ald-1	2	96.607	2	***
Me-2	2	5.492	2	NS
(Totals)			18	***

P. caerulea

Locus	No. of alleles	χ^2	D.F.	p
Pgi-1	4	199.416	15	***
Pgm-1	4	602.579	15	***
G3pdh	3	1060.989	10	***
Pgdh	3	116.918	10	***
Np	3	14.726	10	NS
Mpi-1	4	941.586	15	***

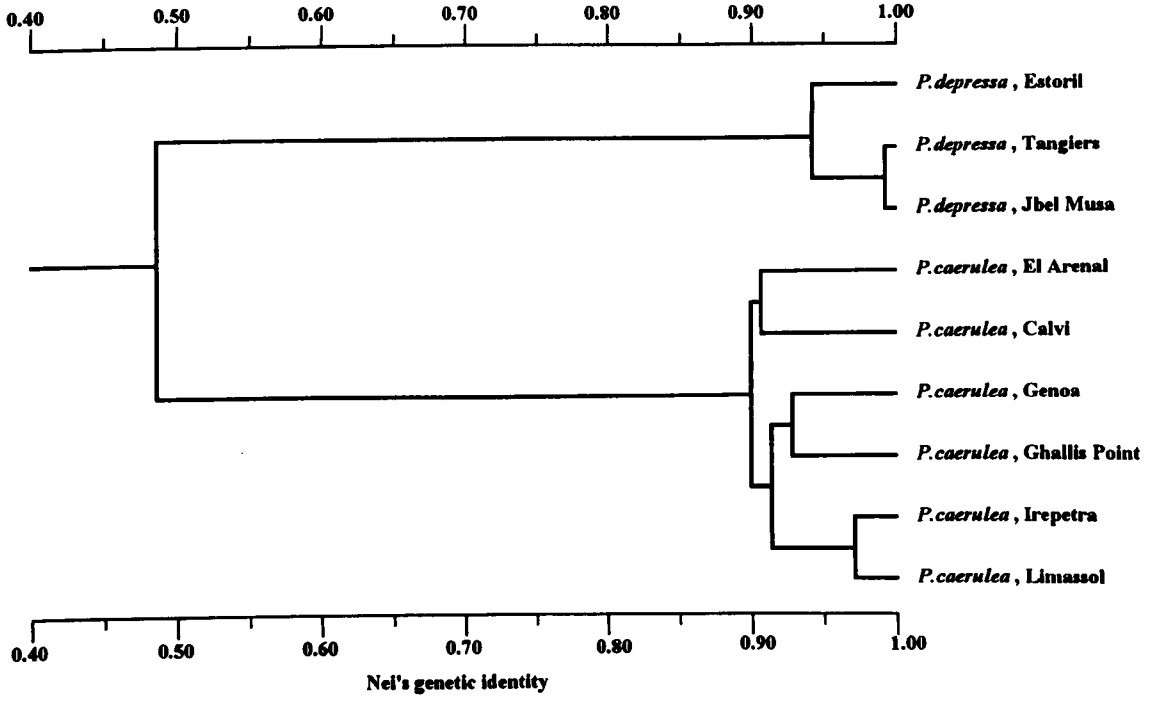


Figure 5.28 - Dendrogram based on a UPGMA cluster analysis of Nei's (1978) genetic identity between the three *P.depressa* samples and the six *P.caerulea* samples.

Table 5.23 - Summary of Chi-squared statistics for the analysis of heterogeneity of allele frequencies over the polymorphic loci between the samples of the same species. Shown are Number of alleles, Chi-squared value, degrees of freedom (D.F.) and probability level (p), where N.S. = not significant.

P. depressa

(Totals)			75	***

5.4 - Discussion

5.4.1 - Conflicting information from multivariate techniques

The results of the multivariate analysis of the morphometric data in this chapter highlight a problem in the use of these techniques, or rather, demonstrate the need for care in interpreting results. The problem arises in those techniques that utilise the group structure of the data in the analysis: such as canonical variate analysis and linear discriminant function analysis (the two will henceforth be referred to in conjunction as canonical discriminant analysis).

Principal component analysis treats the whole data set as a single group and determines the axes in multivariate space that explain the maximum amount of variance in the data set (Krzanowski 1988). The group structure of the data set does not affect the analysis itself. Where different group structures are used (e.g. species or sites) these are merely different ways of looking at the same set of results. Whether or not one finds differentiation of the two species or the nine individual samples using principal component analysis depends on whether large proportions of the variance in the data set are caused by inter-group differences. The relationship between any two points in multivariate space is not affected by the group structure upon which the interpretation of results is based (Krzanowski 1988). The same is not true of canonical discriminant analysis.

Canonical discriminant analysis involves non-orthogonal rotation of axes (See chapter 2) and thus distortion of the relationship between points in multivariate space (Krzanowski 1988). This is important where more than one group structure is used in the analysis of the data set.

Canonical discriminant analysis attempts to maximise the distances in multivariate space between the groups used in the analysis (Krzanowski 1988). In the first canonical discriminant analysis the data set was divided into two groups: *P. depressa* and *P. caerulea* samples. The analysis acts to maximise the distances between these two groups. The resultant group means and canonical coefficients are the product of axis rotation and distortion of the multivariate space (Krzanowski 1988) (See chapter 2). The canonical discriminant analysis was repeated using nine groups, one for each sample site. The analysis now acts to maxi-

mise the distances between these nine groups. The resultant group means and canonical coefficients are again the product of axis rotation and distortion of the multivariate space. In my opinion the problem with this is not that both runs of the analysis distort the relationship between any two points in space, but rather that in all probability they distort it differently. The maximum distance between the two species is unlikely to result in the same relationship between any two points as the maximum distance between the nine sites will. That is that the relationship in space between any two points will be different depending upon the group structure used in the analysis. As the two runs of the canonical discriminant analysis produce different answers, I contend that they are best used to answer different questions.

Separating the data set into the two species is best used to investigate differences in shell size and shape between the two species and to look at variation in shell shape within each of the species as a whole. The problem with this is that if one sample site within a species is markedly different from the others the analysis will not show this, and the differences could reduce the separation obtained between the two species. But if one is looking at the scope for variation in size and shape within a species, then such reduced separation is not a problem but an indication of a wide realised variation in shell size and shape. Breaking the data set down into the nine samples gives information on the variation in shell size and shape of the two species within and between the nine sample sites. As such it is useful in determining whether differences in shape between species are mainly due to variation in the shape of limpets from different sites or to intra-sample variation.

5.4.2 - The relationship between radula length and the shell measures

In the principal component analysis where radula length was included the correlation coefficients between radula length and the shell measures are relatively low and in the eigenvector of principal component 1 the value for radula length is lower than for the other characters. It is worth looking into the possible reasons for this. There is likely to be a strong positive correlation between all the shell measures (i.e. not including radula length) of any data set, even if the presence of two species, or the fact that within a sample there are individuals from a range of microhabitats, means there are allometric elements in the variation in size and shape. This correlation is due to the restrictions on the variation in the relationships between shell measures imposed by shell geometry and the way in which limpet shells grow

(Ekaratne and Crisp 1983, 1984). The analysis of the shell morphology of *P. caerulea* in chapter 4 showed there to be a large component of variation in shell size and shape that is isometric (that is the shell measures increase in a constant ratio), however the relationship between radula length and the shell measures is not such a rigid one, as evidenced by the low correlation coefficients and the first principal component of the analysis that included radula length. The factors that affect shell shape such as time spent clamped down on rock (Moore 1934), and related to this exposure to wave action (Ebling *et al.* 1962; Branch and Marsh 1978) and exposure to air (Ebling *et al.* 1962; Davies 1969), act upon the shell shape as a whole and not on any individual shell measure (Ekaratne and Crisp 1983). The radula length is not linked to the other measures as strongly and can vary much more, relative to shell size, than any of the shell measures can. The factors affecting radula length will in part be the same as those affecting shell shape (Moore 1934; Ebling *et al.* 1962; Davies 1969). But there are other factors that act on radula length and not directly on shell shape. The rock type that is grazed over will affect the rate at which the radula is worn and lost as will the time spent grazing. The dynamic nature of the radula length will also affect the relationship between radula length and shell size. The radula is continually growing at one end and being worn down and lost at the other end. Thus factors that affect the rate at which it grows (e.g. metabolic activity) and the rate at which it is lost (e.g. rock type or time spent feeding) will affect the relationship between radula length and shell size in an individual over time. So if conditions arise where the rate of wear is greater than the rate of replacement of the radula, the ratio of radula length to shell size will decrease. Such a reduction could only occur in one of the shell measures due to shell damage. Clearly then the relationship between radula length and shell size can vary in a individual and will vary between individuals of the same size, to a greater extent than for any of the shell measures with respect to shell size.

The reason for the low correlation between radula length and the shell measures is the fact that the analysis is dealing with two species. Work on the variation, between species, in shell measure ratios and R/C ratios has shown there to be greater variation, between species, in R/C ratios than in the ratios between shell measures (Davies 1969). Coupled with the high importance of radula length in discriminating between *P. depressa* and *P. caerulea* in this study, this obviously implies that the disparate relationships between radula length and shell size of the two species will result in lower correlation coefficients between radula length and

the shell measures and in a deviation from isometry in the first principal component of the analysis where radula length is included.

5.4.3 - Shape variation within and between the two species

Both the principal component analysis and the canonical discriminant analysis show there to be strong differences in shell size and shape between *P. depressa* and *P. caerulea*. Both techniques indicate that *P. depressa* has an apex closer to the anterior of the shell and a greater shell height than *P. caerulea*. Additionally the canonical variate analysis shows *P. depressa* to have a greater shell weight than *P. caerulea*. These differences are sufficient for the two species to be correctly identified in 90% of cases by a linear discriminant function analysis. Where radula length is included the differences between the two species are even more pronounced, with the radula length of *P. depressa* being greater than that of *P. caerulea* of similar size.

Clearly there are differences in shell shape that would enable *P. depressa* from an Atlantic environment to be separated from *P. caerulea* from a Mediterranean environment. Whether these differences in shape would still be evident with a sample of *P. depressa* taken from the Mediterranean is a different matter. Estoril is on the Atlantic coast of Portugal and both Tangiers and Jbel Musa have an environment heavily influenced by the Atlantic. These three locations are exposed to a very different set of environmental conditions, especially tidal regimes, from those experienced by the sites within the Mediterranean. The Mediterranean sites have a much reduced tidal range, which leads to a very different temperature regime for intertidal animals, different exposure to wave action and subject to different predator species in the Atlantic compared to the Mediterranean . The results from this and the previous two chapters show there to be a very large variation in the shell morphology of *P. caerulea* and *P. depressa* and the results of the previous chapter suggest that in *P. caerulea* this variation is largely environmentally induced. There is a wealth of literature relating variation in shell shape and radula length to environmental conditions in patellids (Fischer-Piette 1935, 1939; Comfort 1946; Brian and Owen 1952; Fischer-Piette and Gaillard 1959; Ebling *et al.* 1962; Blackmore 1969a; Davies 1969; Bannister 1975; Branch and Marsh 1978; Branch 1981; Baxter 1982, 1983). With this in mind it is impossible to

predict what affect a Mediterranean environment (such as the Spanish Mediterranean coast) will have on the shell shape of *P. depressa.*

I would suggest therefore, that to try to identify a patellid on the Spanish Mediterranean coastline as *P. depressa* on the basis of shell morphology is very likely to lead to errors in identification. Looking again at the genetic results for the limpets identified as *P. depressa* at Estopona (Spain) by Corte-Real (1992), it is likely that most (if not all) are in fact mis-identified *P. caerulea* , as they show the allele for *P. caerulea* at the *Mdh* locus, the dominant allele for *P. caerulea* at the *Ald* locus and have a higher identity with *P. caerulea* than *P. depressa.* To further highlight the difficulty of identifying *P. depressa,* I have had a worker experienced in the identification of Atlantic limpets (Dr S.J. Hawkins), confidently identify individuals of *P. caerulea* from Malta as *P. depressa* on the basis of morphology and *P. caerulea* from St Tropez and Corsica as *P. depressa* based on soft body characteristics - he was incorrect. I feel that the only way to safely determine the existence of *P. depressa* on the Mediterranean Spanish coast is the use of enzyme electrophoresis or a similar tool to test individuals against individuals of the two species taken from sites where the other clearly does not occur.

5.4.4 - Factors affecting the present distribution of P.depressa and P.caerulea

There is a strong inflow of surface water from the Atlantic into the Mediterranean carrying a recognisable Atlantic planktonic component (Alvarino 1958). This surface water inflow introduces southern rather than boreal species as it is generally a warm water inflow from North Africa (Blanc 1968; Pérès 1967). The hydrological conditions allow greater penetration by Atlantic species along the North African coast than the Spanish coast (Pérès 1967).

The characteristics of the Atlantic inflow into the Mediterranean will have two effects on the genetics and distribution of *P. depressa* in the Mediterranean. Firstly, the current flow along the North African coast allows greater penetration of *P. depressa* along this coast, as evidenced by the *P. depressa* population at Jbel Musa. Secondly, as the inflow is generally of warm water from the African side of the mouth of the Mediterranean, the two samples from Morocco will have a high potential gene flow between them (the current flowing past Tangiers to Jbel Musa). This inflow will also result in no direct current flow between

the Portuguese coast and the Moroccan Mediterranean coast, as evidenced by the restricted gene flow between Estoril and the two sites in Morocco.

The penetration of *P. depressa* along the Spanish coast will probably be heavily affected by the presence of a hydrological barrier in the region of Cabo de Palos (Spain) and Cabo de la Gata (Spain) (See Dando and Southward 1981). The western flow of water along the French and Spanish Mediterranean coast goes offshore in this region, past the Balearics to rejoin the eastward flow (Romanovsky 1955; Bougis 1958; Suau and Vives 1958). This also results in the inflow of Atlantic water along the Spanish coast going offshore and supplying the Morocco coast (Pérès 1967). These current patterns will make the penetration of *P. depressa* past this cut off point around Cabo de Palos very difficult. This hydrological barrier is augmented by a physical barrier in the form of long stretches of sandy coast, unsuitable for limpets, north-east of Cabo de la Gata (Dando and Southward 1981).

The water flow from the Spanish coast to Morocco could also be a mechanism by which divergence between European and Moroccan coast *P. depressa* is prevented, the flow of Atlantic water into the Mediterranean supplying an indirect route for gene flow between the Portuguese and Moroccan coasts.

The strong Atlantic inflow will also affect the distribution of *P. caerulea.* The species does not appear to extend beyond the mouth of the Mediterranean. The inflow would act to prevent larval supply from the Mediterranean out into the Atlantic.

The next stage of the investigation into the relationship between *P. caerulea* and *P. depressa*, and the extent of the penetration into the Mediterranean by *P. depressa*, would be to collect samples of *Patella* from the Spanish Mediterranean coast, particularly from around the hydrological boundary zone. Using biochemical genetics the samples could be tested for the presence of the alleles that are diagnostic between the two species. If *P. depressa* was indeed shown to be present in the Mediterranean beyond the hydrological barrier, then the morphology of those individuals could be examined to see if there is still a clear separation in shell shape, or to look at other shell or soft body characteristics that were valid for discriminating between the two species where they are sympatric.

6 - The genetic and morphological differences between the six species of *Patella* found in the Mediterranean

6.1 - Introduction

The patellids of the Mediterranean show a high level of variation in shell morphology (Sella and Bacci 1971; Christiaens 1973; Sella 1976). Within a species this variation can be seen between limpets from different locations (see Chapter 4), between different zones on the shore (see Chapter 3) and also, importantly, intra-specific variation in morphology in limpets from the same shore zone at a single location (see Chapters 3, 4 & 5). With such high levels of intra-specific variation the identification of limpets can be problematic even for experienced workers (see Christiaens 1983 on Sella 1976).

With the importance of the patellids in the ecology of the littoral zones they inhabit (see Branch 1981; Hawkins 1981a, 1981b; Hawkins and Hartnoll 1983; Hawkins *et al.* 1992) it is clearly important that one be able to correctly identify individuals of this group. If they cannot be accurately separated then the role of the individual species cannot be factored out.

The main aim of this chapter is to provide methods of distinguishing the six species of *Patella* found in the Mediterranean that transcend the high intra-specific variation in morphology. This will be based partly on traditional descriptions of soft body and shell characteristics (see Christiaens 1973), but also on multivariate techniques applied to the shell morphology.

The use of multivariate statistics to differentiate morphologically similar species is a recent but well documented technique (Janson 1987; Janson and Sundberg 1983; Sundberg 1988; Grahame and Mill 1989, 1992; Kristensen and Chrisensen 1989; Grahame *et al.* 1990; Côrte-Real 1992; Takada 1992). It will be applied in this chapter with two major aims in mind: firstly, to characterise samples of the six Mediterranean species of *Patella* using canonical discriminant analysis to determine the differences in shell shape between the species; secondly, to assess the use of a cross-validated linear discriminant function to classify individuals into the correct species group, with the possibility of using the derived function to identify further samples or museum specimens.

The use of biochemical genetics allows the possibility of individuals belonging to a particular species to be ascertained to a very high level of statistical probability (Nei 1972; Thorpe 1979). The presence of loci diagnostic between species is an invaluable tool in the correct identification of individuals collected in the field. This technique will be used to confirm the identity of individuals when there is any doubt about which species it is.

6.2 - Materials and methods

6.2.1 - Collection of samples

Samples of the six species of *Patella* found in the Mediterranean were collected. The details of the sampling sites for each species are given below in Table 6.1. The Irepetra sample contained both infralittoral and mesolittoral *P.caerulea*,whilst all other samples of *P.caerulea* were from the mesolittoral zone only. The other species were collected from whichever zone they occurred in.

Table 6.1 - The source of the samples for the six Mediterranean species of *Patella* in this study. The site, country and number collected are given.

Species	Site	Country	Number
P.aspera	Ghallis Point	Malta	39
	Irepetra	Crete	44
P.caerulea	Isle Rousse	Corsica	99
	St Tropez	France	20
	Ghallis Point	Malta	100
	Irepetra	Crete	55
P.depressa	Estoril	Portugal	100
	Tangiers	Morocco	165
P.ferruginea	Isle Rousse	Corsica	15
P.nigra	Tangiers	Morocco	66
P.rustica	Isle Rousee	Corsica	80
	St Tropez	France	84

At each site a sample of approximately 100 individuals was collected in the mesolittoral zone from above the red algal turf zone that marks the top of the infralittoral fringe. Samples of *P.depressa* were collected from the mid-shore zone on tidally influenced Atlantic shores.

6.2.2 - Qualitative soft body characters

A number of soft body characteristics were recorded for each individual: colour of head tentacles, colour of pallial tentacles, presence of orange base to pallial tentacles, and colour of the foot muscle. The sex of each individual was also recorded, along with the gonad stage (Orton *et al.* 1956) and colour.

6.2.3 - Morphological analysis of shell

For each individual a number of shell parameters were measured to the nearest 0.5mm using vernier callipers (Figure 2.21). The two width measurements give an impression of how ovate the shell is. Differences between the two height measures indicate curvature in the basal plane of the shell. Additionally the shell was weighed (to the nearest 0.01g) and radula length was measured (to the nearest 0.1mm). Shells were labelled and stored for future reference (see chapter 2 for further details).

The twelve separate samples (Table 6.1) used in the analysis of the data were grouped by species (i.e. *P.aspera, P.caerulea, P.depressa, P.ferruginea, P.nigra* and *P.rustica*). The three major techniques used in the analysis of the data were: principal component analysis, canonical variate analysis and linear discriminant function analysis. Further details about these techniques are given in chapter 2.

The analysis was repeated based on shell characters only, that is radula length was excluded from the analysis. This was done for a number of reasons:

1 - If the study came up with shell characteristics that strongly differentiated the samples of the six species of *Patella* then this information could be used to re-examine museum shell collections or archaeological middens. It is unlikely that such shell collections will contain information about the radula length.

2 - To allow for the fact that the exclusion of radula length may improve the differentiation between some species (e.g. if two species have similar relative radula lengths that mask differences in shell shape).

From chapter 2 it is clear that there is little loss of discrimination between groups of *P.caerulea* where radula length is not included, so the exclusion of this character will still allow differentiation between groups based on the remaining seven shell characters.

6.2.4 - S.E.M. analysis of the radula structure

Analysis of the radula structure was carried out on selected stored specimens of each species. The radula was removed from 70% ethanol and air dried. It was cut into short sections and mounted on a stub using double-sided cellotape. Care was taken to ensure the radula was securely positioned, with the basal strip of the radula attached to the cellotape, but radula manipulation was minimised to reduce excess damage to the teeth. The radula was examined using a Phillips S.E.M.

6.2.5 - Electrophoresis

Electrophoresis on the samples was carried out following the methods described in detail in the general materials and methods (Chapter 2). Briefly, for the electrophoretic work the foot muscle tissue of the limpet was used. The muscle was removed from live limpets; only healthy individuals were sampled. The genetics of the samples of limpets was examined using standard horizontal starch gel electrophoresis (following Selander *et al.* 1971; Harris and Hopkinson 1977; Ferguson 1980). Tris citrate pH 8.0 was used as the gel buffer. The gel slices were stained using standard solutions (Shaw and Prasad, 1970; Schaal and Anderson, 1974; Harris and Hopkinson, 1978). Thirteen loci were assayed : *Pgi-1*, *Pgm-1*, *G3pdh*, *Pgdh*, *Np*, *Mpi-1*, *Mdh-1*, *Mdh-2*, *Ald-1*, *Idh-1*, *Idh-2*, *Me-2* and *Got*.

The electrophoresis was used to confirm the identification of individuals where there was some doubt about the species of an individual. This applied particularly to samples of *P. aspera*, *P. caerulea* and *P. depressa*. All individuals from samples of any of these three species had their identities checked electrophoretically. Diagnostic loci were used to confirm that each individual had been correctly identified. Details of such differences between *P. caerulea* and *P. depressa* are given in the previous chapter (see Chapter 5). The *Np* locus could be used to distinguish *P. aspera* from the other two species. *P. aspera* was found to be fixed at the *Np* locus for a slower, and much more heavily staining, allele than the other two species (C.Nobles unpublished).

P. ferruginea, *P. nigra* and *P. rustica* each had sufficiently distinctive morphological and soft body characters that electrophoresis was not needed to confirm the identities of individuals assigned to any of these three species.

6.3 - Results

6.3.1 - Soft body and distinctive shell characteristics

Table 6.2 gives descriptions of the soft body and distinctive shell characters for each of the species studied. The species that can be positively identified on the basis of these descriptions are *P.ferruginea*, *P.nigra* and *P.rustica*.. The information in this table highlights several difficulties in the differentiation of species on the basis of soft body characters:

1 - Although some species have distinctive characters, such as a yellow foot and dark head tentacles in *P.ferruginea*, there are individuals from other species that have similar coloration.

2 - It is quite easy to distinguish some of the species on the basis of soft body coloration and characteristics (C.Nobles *pers. obs.*). The problem is that the basis on which one does differentiate species is generally a subtle combination of character traits that is very difficult to quantify and even harder to convey in written descriptions. Thus the description of soft body coloration is of limited use in conveying to other workers how one can differentiate between species.

A number of the species have distinctive shell character and soft body traits that enable one to identify individuals as members of a particular species. Given here are only those characteristics that I consider to be unambiguous in the identification of individuals:

1 - *P.ferruginea* - A very thick, strong heavily ribbed shell. The 25 to 50 external ribs are large, pronounced and result in a crenulated shell margin. The shell exterior tends to be ashen in colour with concentric red/brown rings. The shell interior is white nacre sometimes with a bluish tint, there is a black to brown/black border to the internal circumference of the shell, and a brown ring where the soft tissue was attached.

Table 6.2 - Descriptions of the soft body characteristics and distinctive shell character for the six species of *Patella* found in the Mediterranean.

Species	**Foot colour**	**Head tentacle colour**	**Pallial tentacle colour**	**Gonad colour**	**Shell characters**
P.aspera	Light cream to grey. Majority yellow or orange. A few have a lighter centre.	Translucent to dark grey/black. Majority light grey.	Chalky white, occasional black fringe to pallial margin.	Male:Cream to orange Female:Red/Brown to Brown.	
P.caerulea	Light cream to very dark grey. Centre normally lighter - light cream to dark orange.	Dark grey/black through grey to translucent.	White. Some stark white . Some with orange bases, mainly in infralittoral individuals.	Male: Cream to orange-cream Female: Olive to dark brown	
P.depressa	Cream to dark grey. Centre often lighter - cream to dark orange. Orange /brown common.	Dark grey/black through grey to translucent. Grey common.	Stark white	Male: Cream to orange Female:Olive to dark brown	
P.ferruginea	Yellow or light yellow.	Dark grey to black	White	Not available - only neuters found.	Thick heavy shell, heavily ribbed, crenulated margin.
P.nigra	Black or dark grey.	Head dark black, black head tentacles		Male: Female:	Ovate, anterial apex, depressed shell.
P.rustica	Light cream to dark grey, majority light to dark grey. Centre sometimes lighter.	Translucent to dark grey. Majority grey to light grey.	Transparent	Male: Cream to orange Female:Olive brown to brown	High shell, external radial ribs with black nodules.

2- *P.nigra* - A thin, oval shell with a smooth circumference, very elongate, narrow across the apex with greatest width towards then shell posterior. Shell apex sub-central, located well towards the shell anterior (anterior to apex distance approximately 1/3 of shell length). A black or dark grey foot and coupled with a dark head and head tentacles.

3 - *P.rustica* - This is the only Mediterranean species to have transparent pallial tentacles. The distinguishing shell feature of this species is the exterior radial ribs on which there are numerous black nodules. In some shells worn these nodules have been worn down by wave action, but are still evidenced by black marks where the nodules originally were.

6.3.2 - Radula structure

Plates 6.1 and 6.2 show the radula structure of *P.aspera*. The radula formula is 3+1+4+1+3 (Plate 6.1). The pluricuspid tooth has four main cusps (Plate 6.2), the two central cusps are the largest and are of approximately equal size. The internal cusp is smaller than the two central cusps. The smallest cusp lies outside the main external cusp (on the left in this plate) and is considerably smaller than the others.

Plates 6.3 and 6.4 show the radula structure of mesolittoral and infralittoral *P.caerulea*. There are no apparent differences in radula structure between limpets from the two different shore levels. The radula structure of *P.caerulea* is 3+1+4+1+3 (Plates 6.3 and 6.5). The pluricuspid tooth has three main cusps (Plates 6.4 and 6.6). The central cusp in the largest followed by the main external cusp with the internal cusp being the smallest; this is in agreement with other workers (Fischer-Piette 1935; Evans 1953). There does appear to be a fourth, minor cusp that lies outside the main external cusp in a similar position to the fourth cusp in *P.aspera*. This fourth cusp is not as pronounced as it is in *P.aspera*. It was found in the radula of other *P.caeulea* examined and is evident in the drawings of the pluricuspid tooth of *P.caerulea* in Fischer-Piette and Gaillard (1959)

The radula structure of *P.depressa* is 3+1+4+1+3 (Plate 6.7). The pluricuspid tooth has three main cusps (Plate 6.8). The central cusp is the largest and the internal cusp the smallest. The central cusp is narrower than in *P.aspera* or *P.caerulea* and the internal side of this cusp is shorter than the external side.

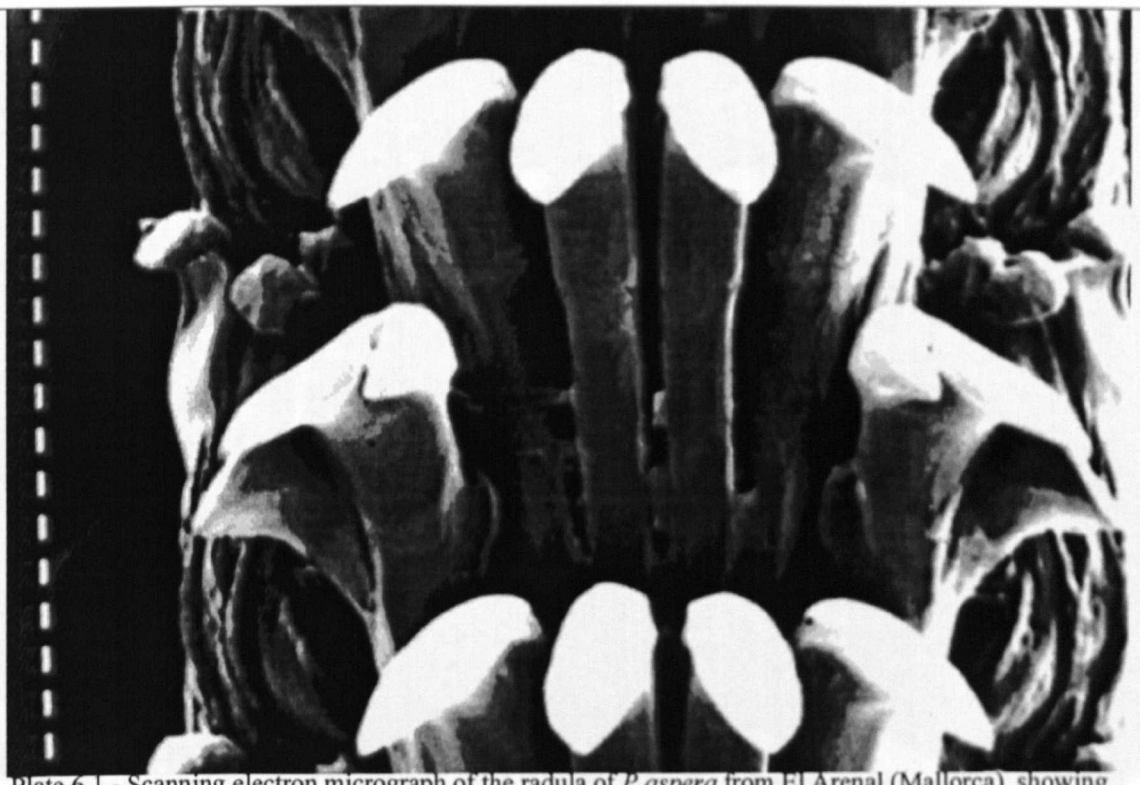


Plate 6.1 - Scanning electron micrograph of the radula of *P.aspera* from El Arenal (Mallorca), showing marginal, pluricuspid and unicuspid teeth from a single row of teeth. Scale marker = 10 μm.

Plate 6.2 - Scanning electron micrograph of the radula of *P.aspera* from El Arenal (Mallorca), showing a pluricuspid tooth. Scale marker = 1.6 μm.

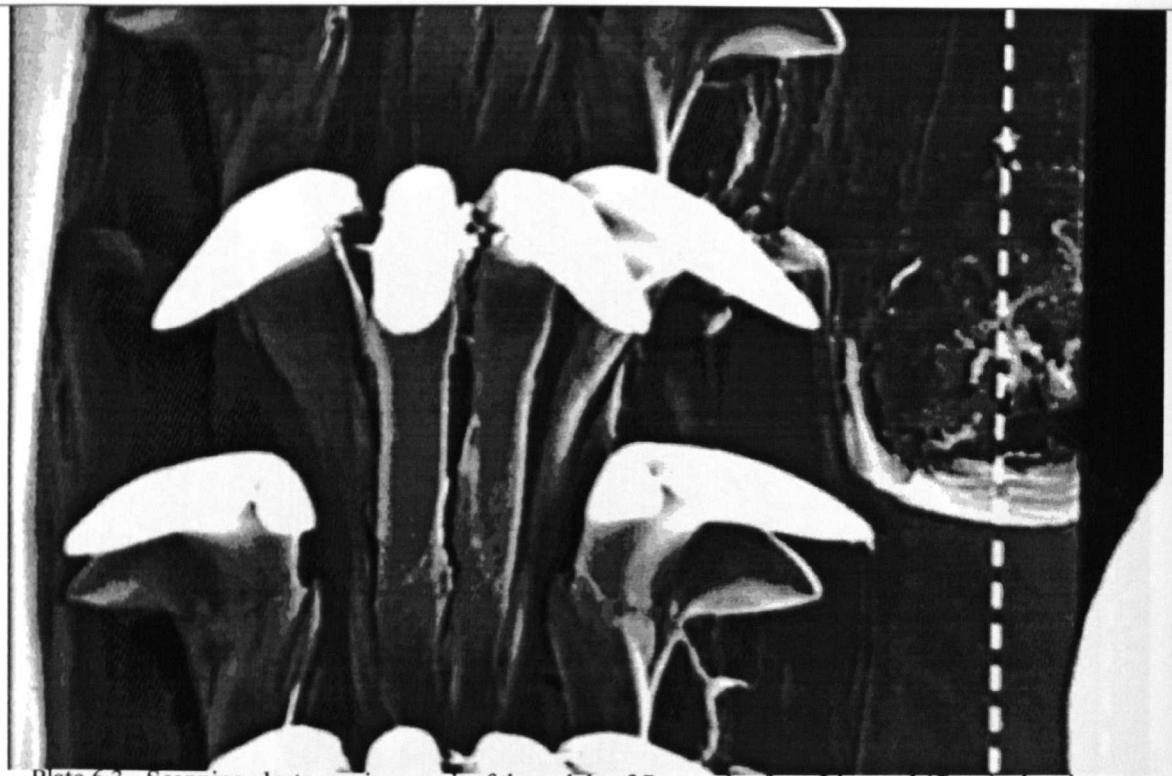


Plate 6.3 - Scanning electron micrograph of the radula of *P.caerulea* from Limassol (Crete), showing marginal, pluricuspid and unicuspid teeth from a single row of teeth. Scale marker = 10 μm.

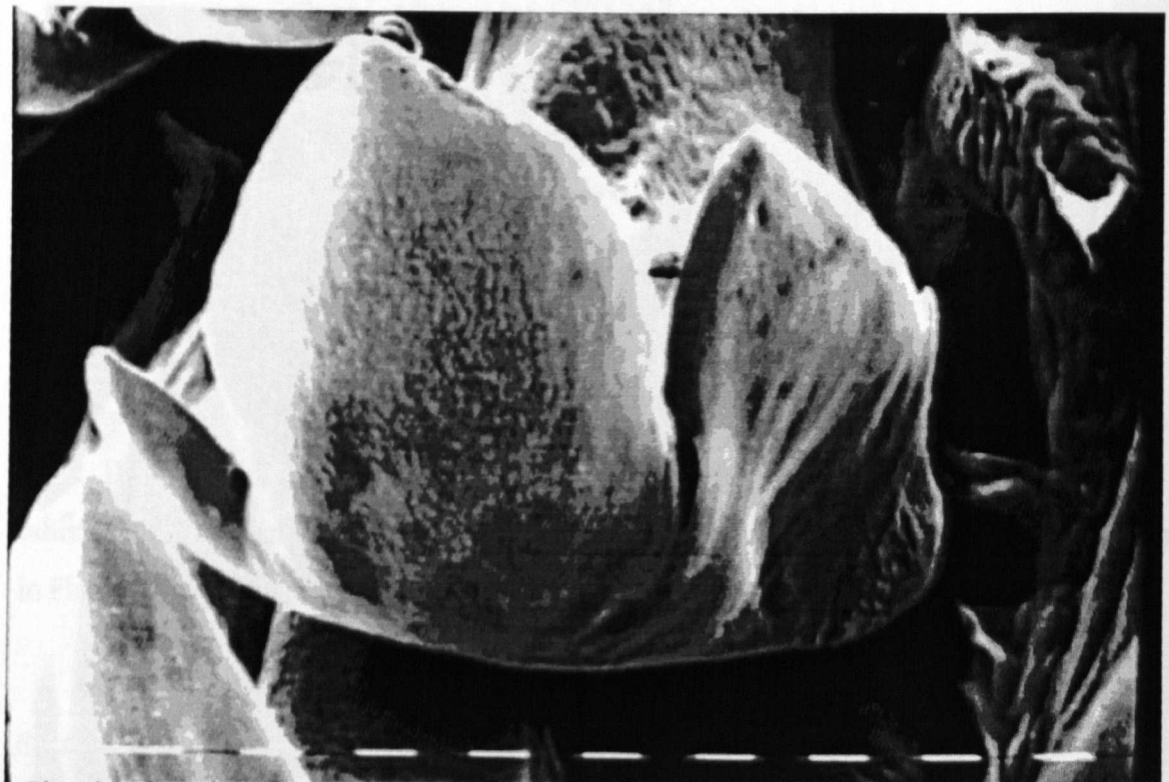


Plate 6.4 - Scanning electron micrograph of the radula of *P.caerulea* from Limassol (Crete), showing a pluricuspid tooth. Scale marker = 1.6 μm.

Plate 6.5 - Scanning electron micrograph of the radula of *P.caerulea* from the infralittoral zone at Limassol (Crete), showing marginal, pluricuspid and unicuspid teeth from a single row of teeth. Scale marker = 10 μm.

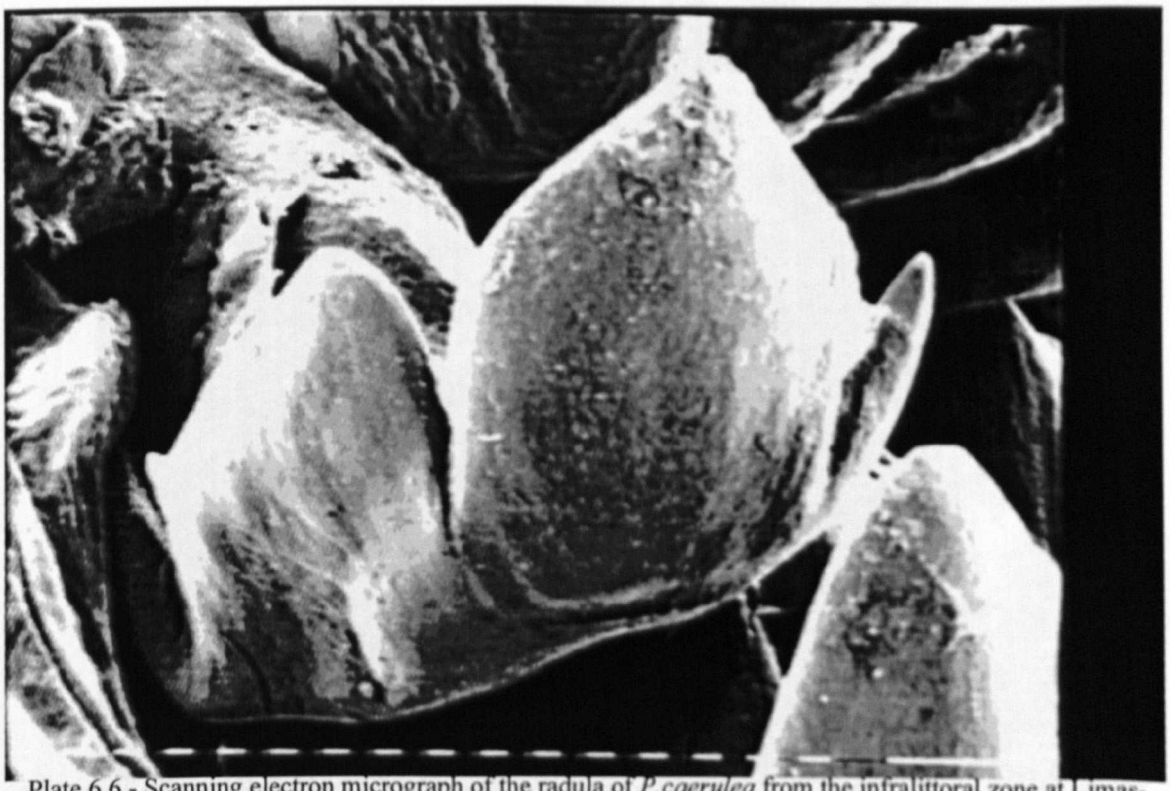


Plate 6.6 - Scanning electron micrograph of the radula of *P.caerulea* from the infralittoral zone at Limassol (Crete), showing a pluricuspid tooth. Scale marker = 1.6 μm.

Plate 6.7 - Scanning electron micrograph of the radula of *P.depressa* from Tangiers (Morocco), showing marginal, pluricuspid and unicuspid teeth from a single row of teeth. Scale marker = 10 µm.

Plate 6.8 - Scanning electron micrograph of the radula of *P.depressa* fromTangiers (Morocco), showing a pluricuspid tooth. Scale marker = 1.6 µm.

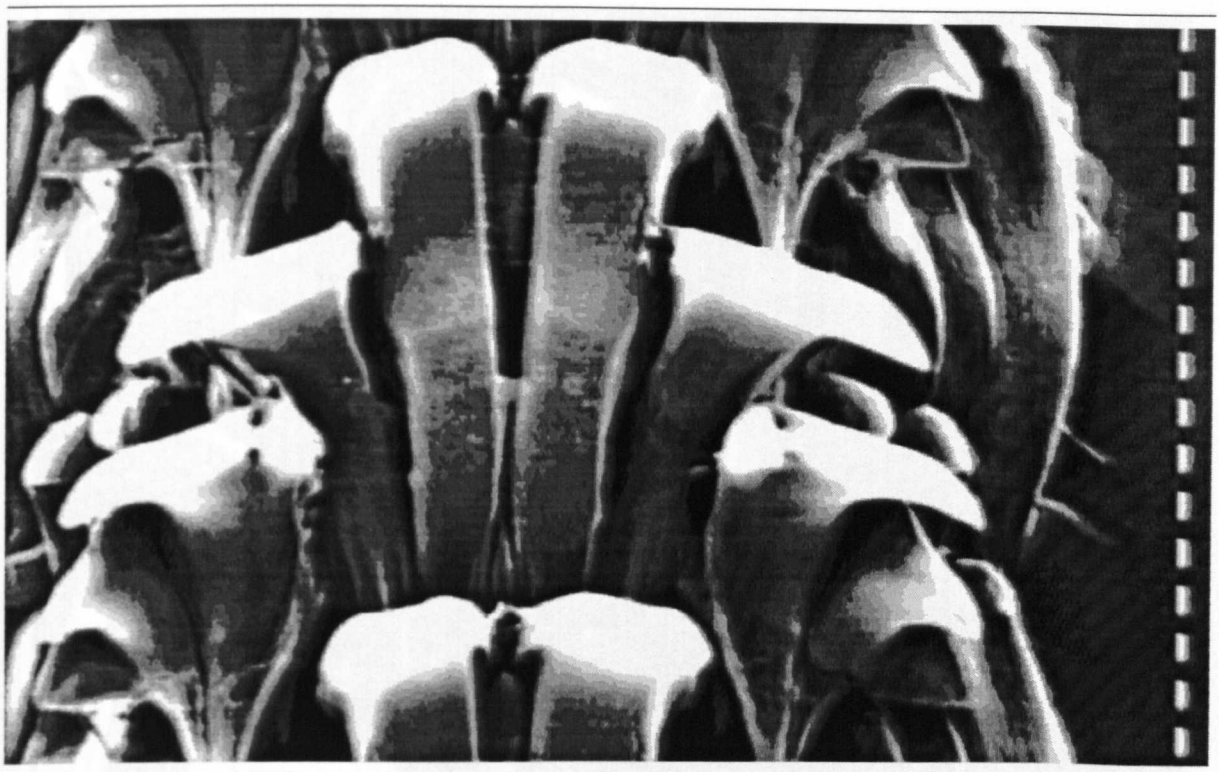


Plate 6.9 - Scanning electron micrograph of the radula of *P.nigra* from Tangiers (Morocco), showing marginal, pluricuspid and unicuspid teeth from a single row of teeth, note the presence of a racidian tooth in the centre of the row of teeth. Scale marker = 10 μm.

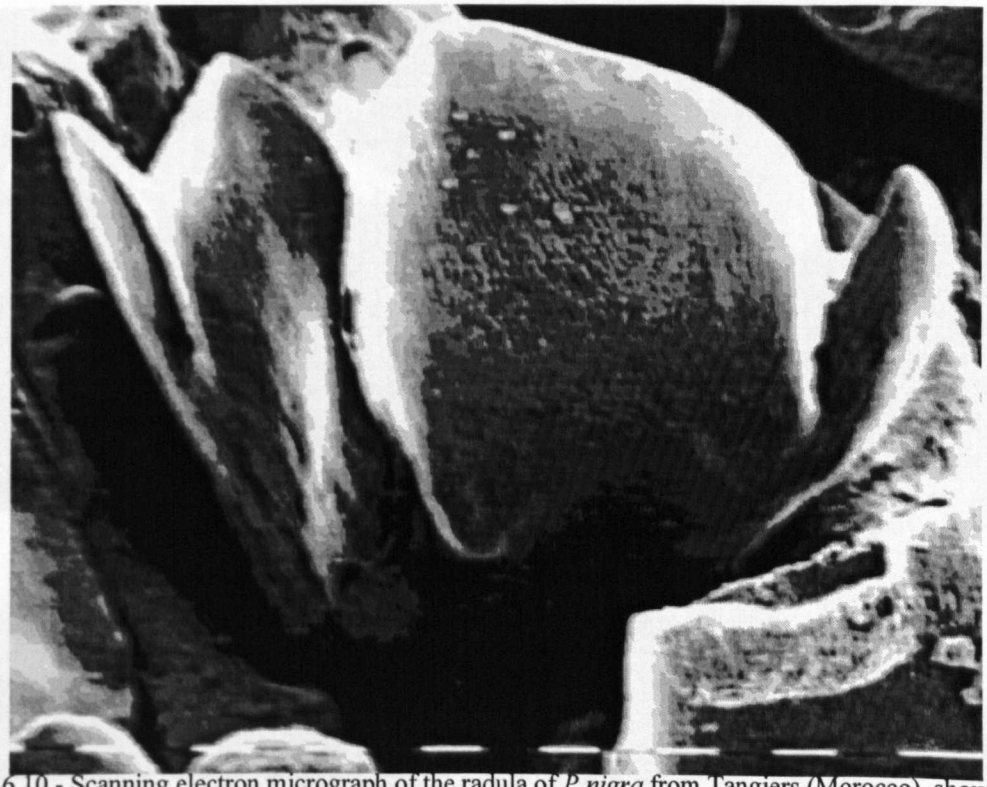


Plate 6.10 - Scanning electron micrograph of the radula of *P.nigra* from Tangiers (Morocco), showing a pluricuspid tooth. Scale marker = 1.6 μm.

Plate 6.11 - Scanning electron micrograph of the radula of *P.nigra* from Tangiers (Morocco), showing the pluricuspid tooth and the layout of the marginal teeth. Scale marker = 1.6 μm.

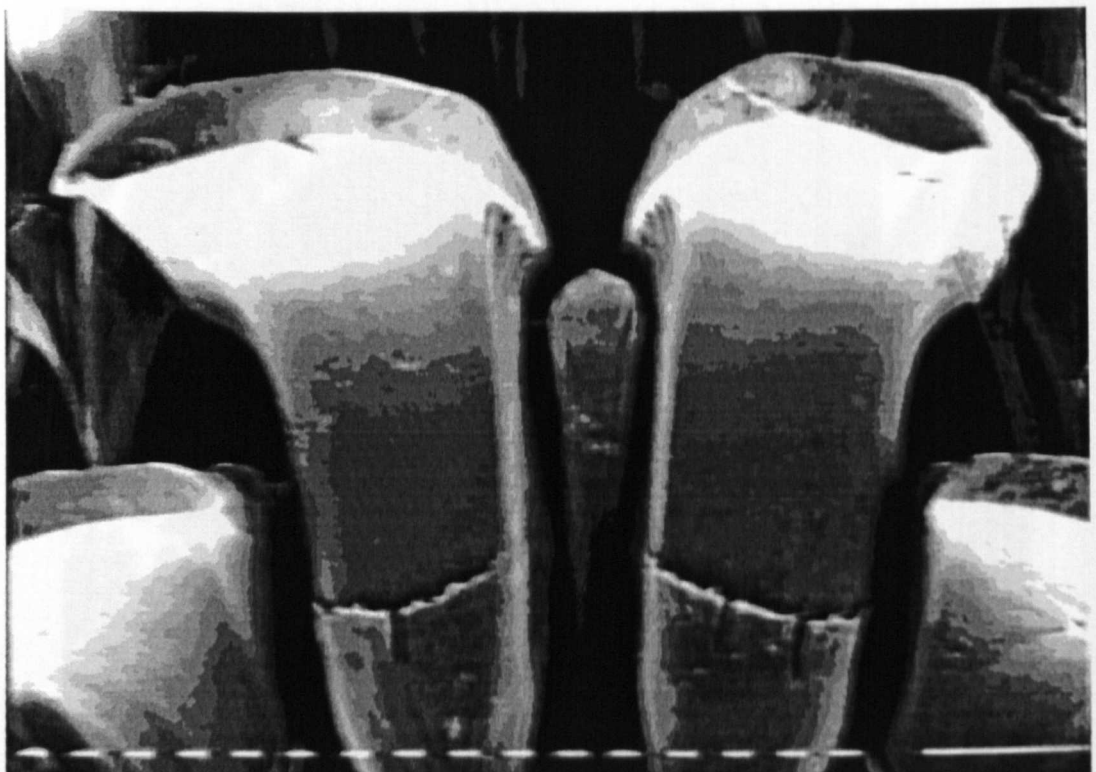


Plate 6.12 - Scanning electron micrograph of the radula of *P.nigra* from Tangiers (Morocco), showing a racidian tooth positioned between the two internal unicuspid teeth. Scale marker = 1.6 μm.

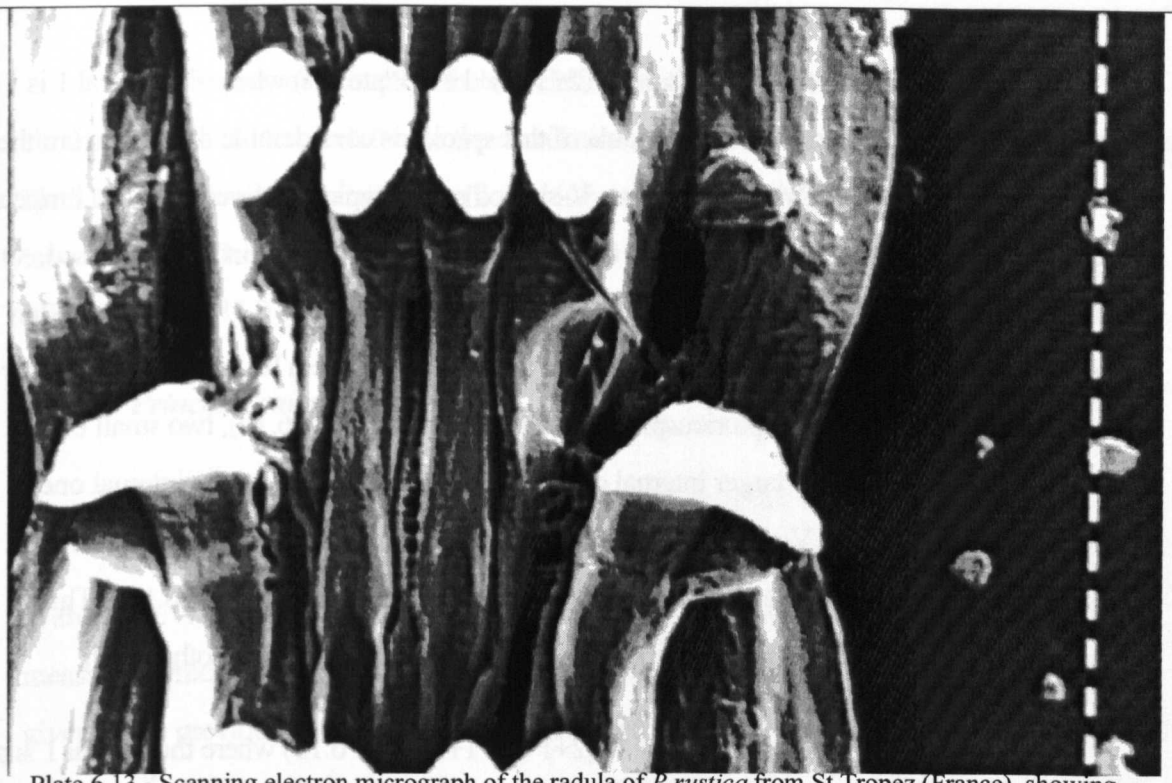


Plate 6.13 - Scanning electron micrograph of the radula of *P.rustica* from St Tropez (France), showing marginal, pluricuspid and unicuspid teeth from a single row of teeth. Note the central rachidian tooth. Scale marker = 10 μm.

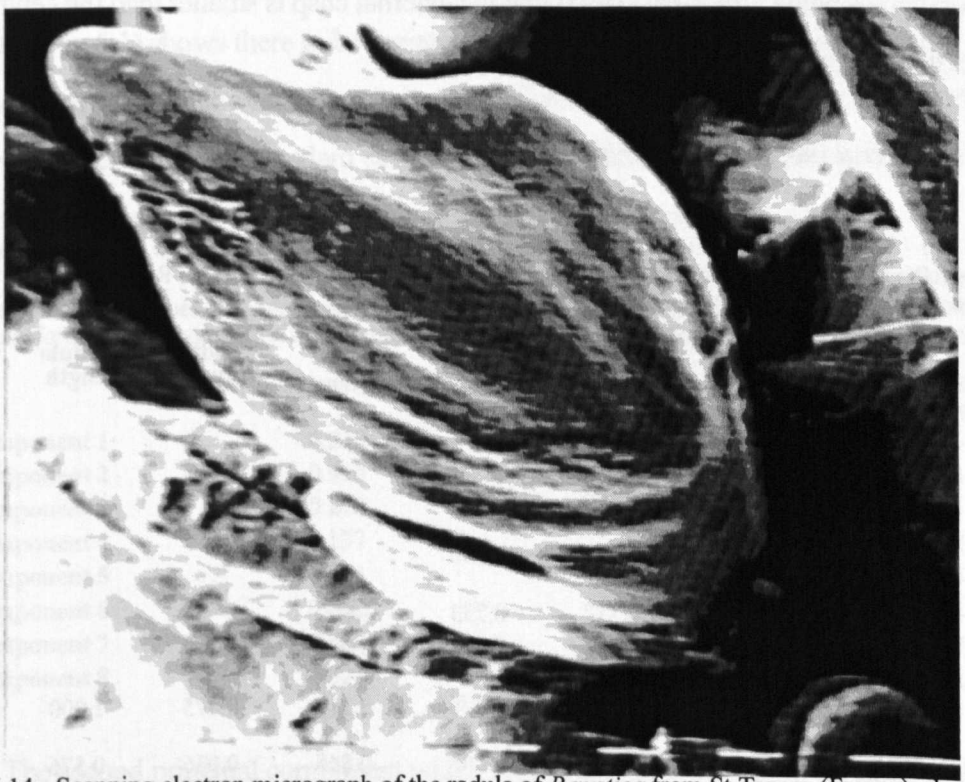


Plate 6.14 - Scanning electron micrograph of the radula of *P.rustica* from St Tropez (France), showing a pluricuspid tooth. Scale marker = 1.6 μm.

The radula structure of *P.nigra* is 3+1+(2+1+2)+1+3 (Plate 6.9) where the central 1 is the position of the rachidian tooth. The radula of this species is considerable different from the other five species in this study. *P.nigra* has a V-shaped radula typical of South African limpets (Koch 1949), whilst in all the other species the teeth, within a row, are horizontally aligned. Of the three marginal teeth, the external one lies below the other two (Plate 6.11). The two external unicuspid teeth lie below the internal ones and the pluricuspid teeth lie below the two external unicuspids (Plate 6.9). The pluricuspid tooth has four cusps (Plate 6.10), two small cusps situated either side of the two larger internal cusps. Of the two central cusp the internal one is the largest. Apart from the V-shaped radula the other feature that distinguishes this species is the large rachidian tooth that lies between the two central unicuspid teeth (Plate 6.12). This tooth is also found in *P.ferruginea* and *P.rustica* but is smaller relative to the other teeth.

P.rustica has a radula formula of 3+1+(2+1+2)+1+3 (Plate 6.13) where the central 1 is a racidian tooth. The rachidian tooth is smaller than in *P.nigra*, but still has a definite cusp. The teeth within a row are aligned horizontally. The pluricuspid tooth has a dominant central cusp (Plate 6.14). Fischer-Piette (1935) states that the external cusp is poorly developed, this study, however, agrees with Côrte-Real (1992) that the external cusp is smaller than the central one and it is the internal cusp that is poorly developed.

6.3.3 - General morphological results

Table 6.3 - Correlation coefficients for data comparing samples of the six *Patella* species. Data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize variance. Radula length is included in the analysis.

	Length	Width (max)	Height (width)	Height (length)	Apex	Width (apex)	Radula length	Cube root of weigth
Length	1.000							
Width (max)	0.957	1.000						
Height (width)	0.816	0.775	1.000					
Height (length)	0.798	0.753	0.956	1.000				
Apex	0.886	0.863	0.727	0.733	1.000			
Width (apex)	0.967	0.967	0.801	0.777	0.901	1.000		
Radula length	0.483	0.466	0.697	0.676	0.317	0.475	1.000	
Cube root of weight	0.961	0.926	0.890	0.868	0.845	0.936	0.576	1.000

As in the previous chapters the correlation coefficients between radula length and the shell characters are low, relative to the correlation coefficients between the shell characters (Table 6.3). The lowest correlation coefficient is between radula length and anterior to apex distance (0.317). The reasons for this are examined in the previous chapter (Chapter 5 Sections 5.3.2 & 5.4.2).

6.3.4 - Principal component analysis

The first principal component accounts for 81.6% of the variance in the data set (Table 6.4). There is unequal weighting in the elements of the eigenvector of principal component 1 (Table 6.5). The radula length element contributes less to this component than do the shell measures, which all contribute approximately the same amount. An explanation for this is given in the previous chapter (Chapter 5 Sections 5.3.3 & 5.4.2). The fact that the weightings of the shell measures in the eigenvector are approximately equal means that principal component 1 can be regarded as an indication of isometric variation in shell size, positively correlated with radula length. Assuming the first principal component to be an isometric size vector for shell size the distribution of group means for the samples of the six species along principal component 1 (Fig 6.1) shows there to be variation in mean shell size between the species. *P.aspera* and *P.rustica* have the lowest mean shell size, whilst *P.ferruginea* and *P.nigra* have the largest mean shell size. The wide standard deviation for the *P.ferruginea* sample is a result of the large range of shell sizes in this sample and the small sample size.

Table 6.4 - Eigenvalues for data comparing samples of the six *Patella* species. Data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize variance. Radula length is included in the analysis.

	Eigenvalue	Percentage of variance	Cumulative percentage
Component 1	6.530	81.6	81.6
Component 2	0.910	11.4	93.0
Component 3	0.278	3.5	96.5
Component 4	0.137	1.7	98.2
Component 5	0.054	0.6	98.9
Component 6	0.042	0.5	99.4
Component 7	0.027	0.3	99.7
Component 8	0.022	0.3	100.0

The second principal component which accounts for 11.4% of the variance is strongly dominated by the radula length element,that is negatively correlated with all the other elements except the two shell height measures. The group means of *P.depressa* and *P.rustica* are signifi-

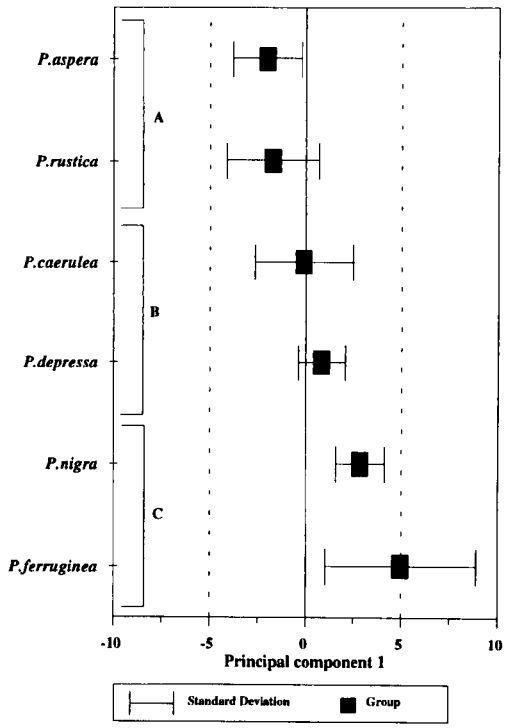


Figure 6.1 - Distribution of the group means of the samples of the six species of *Patella* along principal component 1, with standard deviations shown. There are significant differences between groups ($F=83.3$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data.

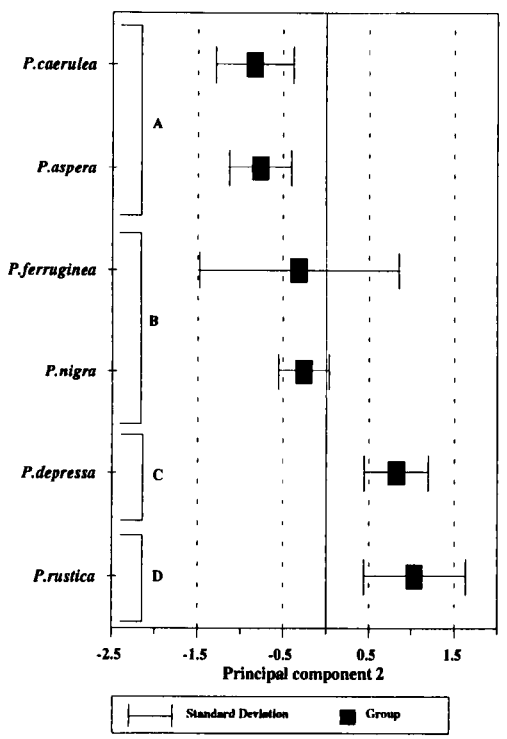


Figure 6.2 - Distribution of the group means of the samples of the six species of *Patella* along principal component 2, with standard deviations shown. There are significant differences between groups ($F=607.51$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data.

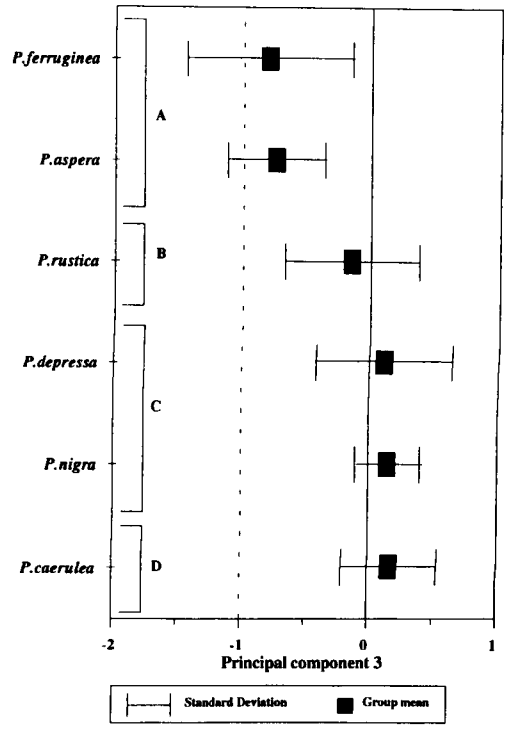


Figure 6.3 - Distribution of the group means of the samples of the six species of *Patella* along principal component 3, with standard deviations shown. There are significant differences between groups ($F=73.05$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data.

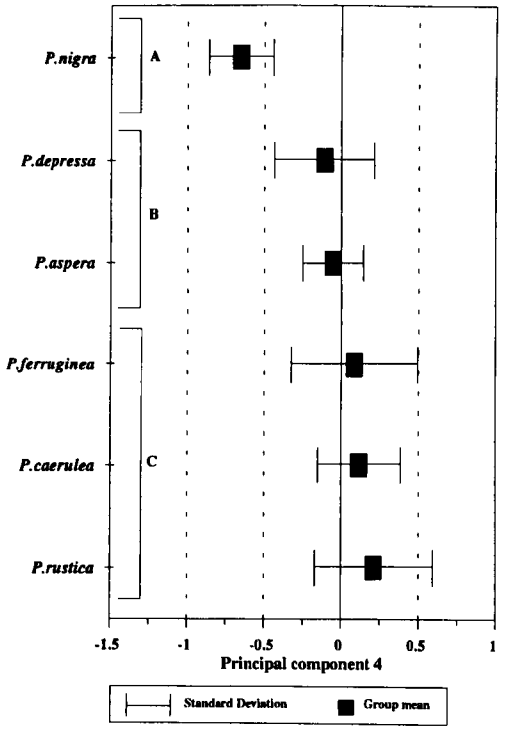


Figure 6.4 - Distribution of the group means of the samples of the six species of *Patella* along principal component 4, with standard deviations shown. There are significant differences between groups ($F=95.9$, $p<0.001$). The groupings from an SNK test are shown, groups with the same letter are not significantly different from each other. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data.

cantly larger than those for the other four species along the second principal axis (Fig 6.2). With high groups means along this axis the relative radula length is greater in *P.rustica* and *P.depressa* than in the other species; the relative radula length in *P.rustica* was significantly longer than in *P.depressa*. *P.caerulea* and *P.aspera* show the smallest relative radula length. It should be noted that the *P.caerulea* sample included infralittoral *P.caerulea* from Irepetra. The radula length apparently related to the shore zone inhabited this would contribute to the *P.caerulea* sample having the lowest group mean for principal component 2.

Table 6.5 - Eigenvectors for data comparing samples of the six *Patella* species. Data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardize variance. $P^{-1/2}$ is the predicted score for each element in principal component 1 if principal component 1 is an isometric size vector. Radula length is included in the analysis.

	Principal component 1	Principal component 2	Principal component 3	Principal component 4
% of variance	81.6	11.4	3.5	1.7
Length	0.377	-0.198	0.185	-0.186
Width (max)	0.368	-0.230	0.343	-0.232
Height (width)	0.361	0.277	-0.436	-0.192
Height (length)	0.355	0.272	-0.562	-0.043
Apex	0.346	-0.359	-0.183	0.814
Width (apex)	0.374	-0.225	0.242	-0.007
Radula length	0.245	0.759	0.499	0.333
Cube root of weight	0.382	-0.038	0.030	-0.313
$P^{1/2}$	0.353			

The third principal component, 3.5% of the variance in the data set, is a contrast between a positive radula length element and negative elements for both the shell height measures (Table 6.5) . *P.ferruginea* and *P.aspera* samples have significantly lower means than the other samples along the third principal component axis (Fig 6.3). This would indicate that relative to individuals of the same size from the other species, *P.ferruginea* and *P.aspera* will have a shorter radula and lower shell height.

The fourth principal component is dominated by a positive apex to anterior distance (Table 6.5). This component accounts for 1.7% of the variance in the data set. The lower group mean for *P.nigra* along this axis (Fig 6.4) indicates that individuals of *P.nigra* will tend to have an apex closer to the anterior of the shell, when compared with individuals of the same size of the other species.

The bivariate plot of principal component 1 against principal component 2 (Fig 6.5a & 6.5b) shows the significant differences in mean shell size for the different species (assuming

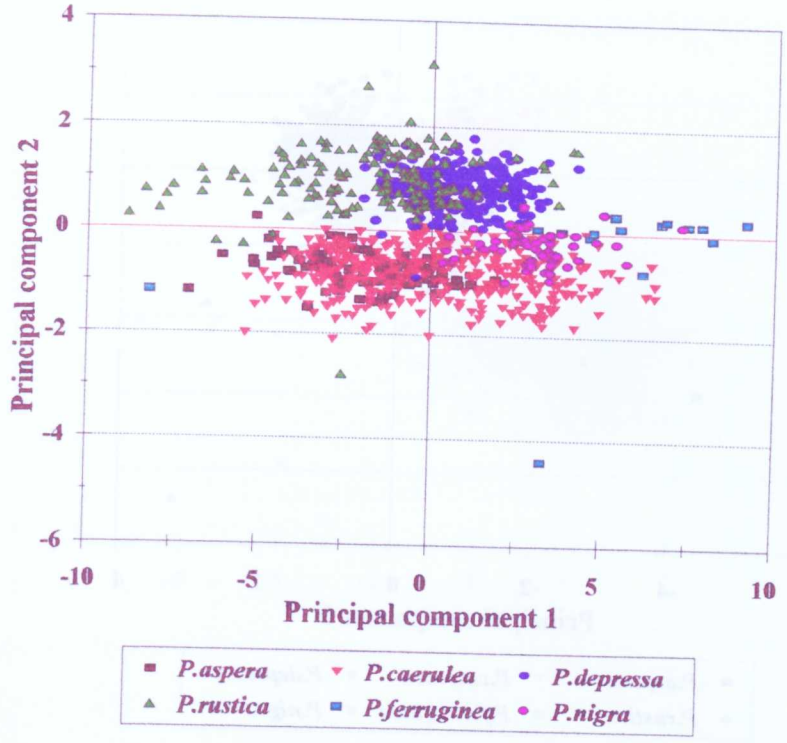


Figure 6.5a - Plot of principal component 1 against principal component 2 for samples of the six species of *Patella*. The data are differentiated by species. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data. The data are separated by species, and individual scores plotted.

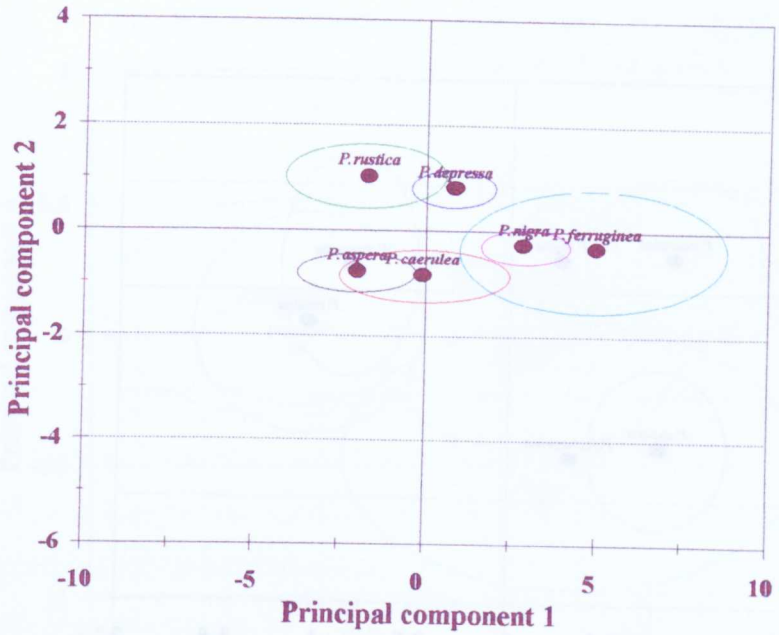


Figure 6.5b - Plot of principal component 1 against principal component 2 for samples of the six species of *Patella*. The data are differentiated by species. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data. The data are separated by species, with group centroids and standard deviations shown.

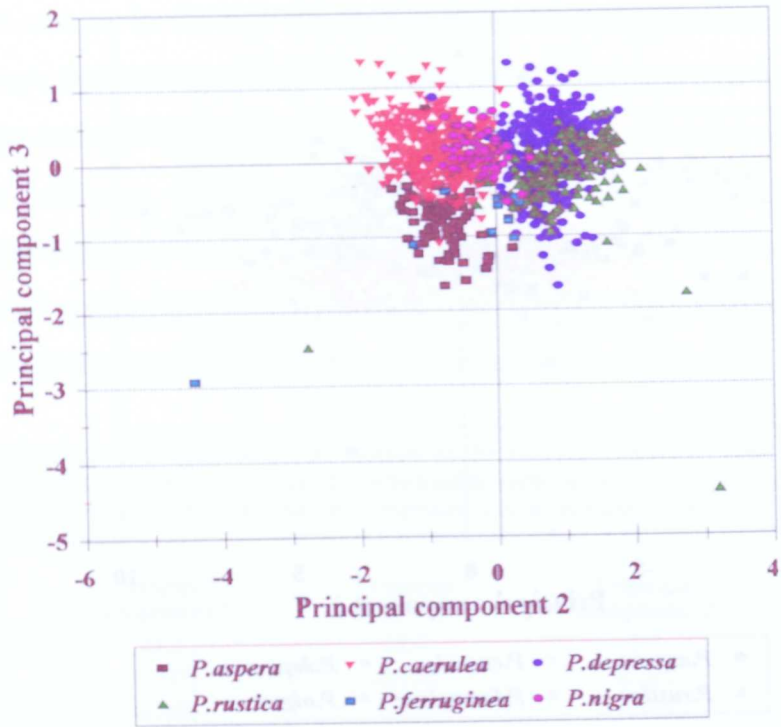


Figure 6.6a - Plot of principal component 2 against principal component 3 for samples of the six species of *Patella*. The data are differentiated by species. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data. The data are separated by species, and individual scores plotted.

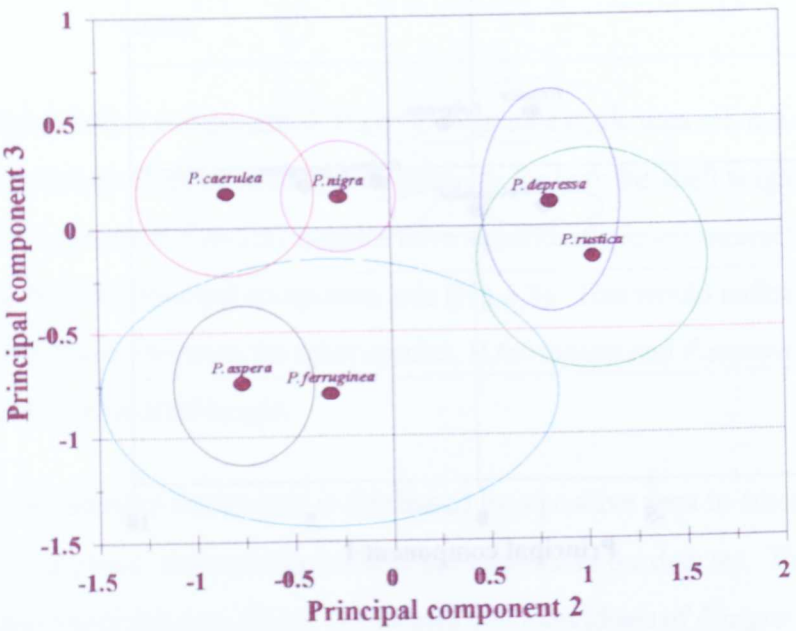


Figure 6.6b - Plot of principal component 2 against principal component 3 for samples of the six species of *Patella*. The data are differentiated by species. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data. The data are separated by species, with group centroids and standard deviations shown.

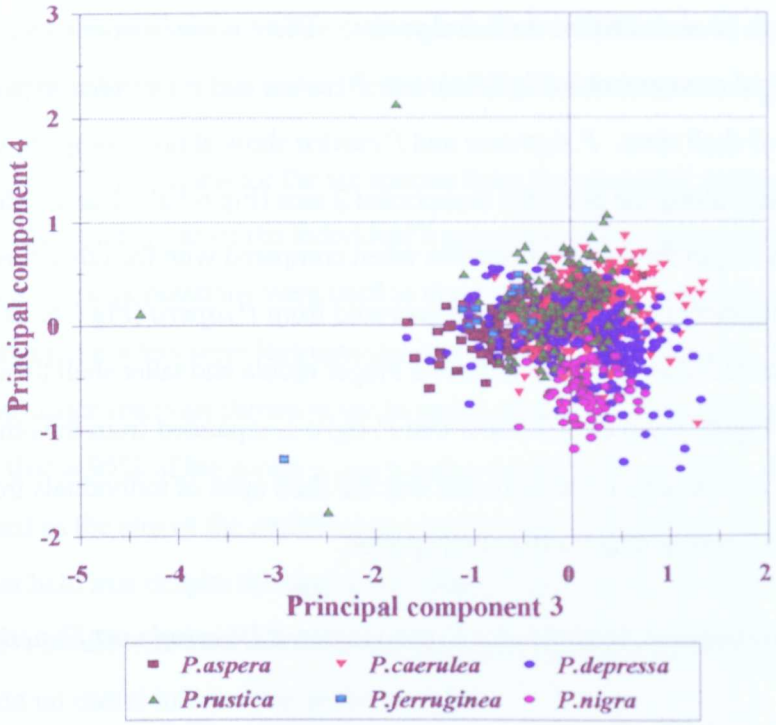


Figure 6.7a - Plot of principal component 3 against principal component 4 for samples of the six species of *Patella*. The data are differentiated by species. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data. The data are separated by species, and individual scores plotted.

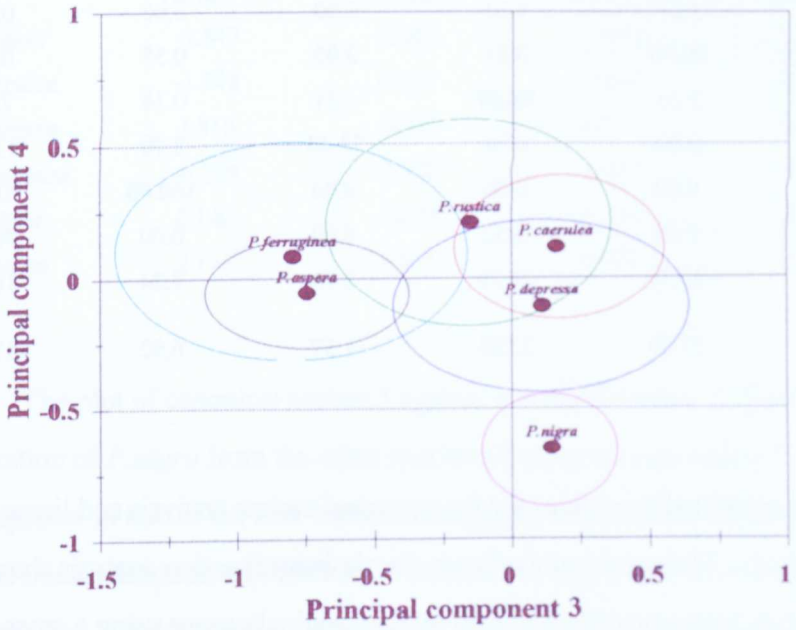


Figure 6.7b - Plot of principal component 3 against principal component 4 for samples of the six species of *Patella*. The data are differentiated by species. The data have been $\log_{10}(x+1)$ transformed and a correlation matrix used to standardise the data. The data are separated by species, with group centroids and standard deviations shown.

principal component 1 to be an isometric shell size vector). There is considerable overlap of the groups along principal component 1 (Fig 6.5a) with *P.rustica* and *P.caerulea* in particular showing a large range of shell sizes. *P.depressa* and *P.rustica* show almost complete separation from the other groups along the principal component 2 axis (Fig 6.6a), clearly indicating a relatively longer radula length for these two species when compared with the other species. The third principal component clearly separates *P.caerulea* from *P.aspera* (Fig 6.6a & 6.6b) indicating that individuals of *P.caerulea* tend to have a longer radula and taller shell than *P.aspera* of the same size. Figures 6.7a & 6.7b show that *P.nigra* is separated from the other species along principal component 4; this indicates that the shell apex of individuals from this sample tends to be more anteriar than for the other species.

6.3.5- Canonical Discriminant Analysis and Cross-validated Discriminant Function Analysis

Table 6.6 - Linear discriminant function using cross-validation showing percentage of individuals from the six species of *Patella* samples correctly assigned to their original groups. The data are differentiated by site. Radula length is included in the analysis.

From SITE	*P.aspera*	*P.caerulea*	*P.depressa*	*P.ferruginea*	*P.nigra*	*P.rustica*
P.aspera	95.18	3.61	1.20	0.00	0.00	0.00
P.caerulea	8.03	**86.70**	0.83	3.05	0.55	0.83
P.depressa	0.00	2.26	**88.68**	1.51	0.38	7.17
P.ferruginea	13.33	0.00	0.00	**73.33**	0.00	13.33
P.nigra	0.00	0.00	0.00	0.00	**100.00**	0.00
P.rustica	3.68	0.00	5.52	0.00	0.00	**90.80**
% allocated to group	12.17	33.79	26.79	2.73	7.24	18.05
% of data set	8.71	37.89	27.81	1.57	6.92	17.10

The data set was separated by species for the canonical variate analysis and linear discriminant function analysis. The results of the linear discriminant function analysis show a high percentage of limpets were correctly assigned to their original groups using a cross-validated linear discriminant function (Table 6.6). The highest percentage assignment was 100% for *P.nigra* and the lowest 73.33% for *P.ferruginea*. This low percentage for *P.ferruginea* is probably due to the low sample size for this species, not enabling the analysis to build a clear

'picture' of the shell shape. Clearly though there are differences in shell shape and radula length between species that are sufficient to differentiate them to a high degree.

The group means for the six species from the canonical variate analysis are given in Table 6.7. Bivariate plots of the individual's canonical scores, group means and 95% confidence intervals of the populations were used to determine which canonical variate and hence which measured parameters were instrumental in separating the six species. It should be noted that the confidence intervals shown in the bivariate plots are 95% confidence interval for the population; that is 95% of the group is predicted to lie inside these limits. This confidence measure was used as the aim of the multivariate analysis was to find differences in shape between species that held true despite the high intra-specific variation in morphology displayed by limpets. The 95% confidence intervals of the group means are considerably smaller and are not used as they add no useful information to the analysis.

Table 6.7 - Group means of the first five canonical variates for the samples of the six species of *Patella* samples are differentiated by species. The data have been $\log_{10}(x+1)$ transformed and standardized. Radula length is included in the analysis.

SITE	Canonical variate 1	Canonical variate 2	Canonical variate 3	Canonical variate 4	Canonical variate 5
P.aspera	-1.547	0.162	-2.224	0.555	-0.321
P.caerulea	-1.948	0.451	0.498	-0.088	0.031
P.depressa	1.816	-0.374	0.531	0.453	-0.063
P.ferruginea	-0.239	-2.278	-1.572	0.583	2.300
P.nigra	0.140	-4.609	-0.311	-0.734	-0.184
P.rustica	2.115	1.602	-0.563	-0.580	0.060

The plot of canonical variate 1 against canonical variate 2 (Fig 6.8a & 6.8b) shows clear separation of *P.nigra* from the other species along canonical variate 2. A clear separation is to be expected in one of the first variates for a group whose members are always correct assigned by the linear discriminant function. Canonical variate 2 also acts to separate *P.rustica* from *P.depressa*. *P.rustica* and *P.depressa* are separated from *P.aspera* and *P.caerulea* along canonical variate 1. The low sample size of *P.ferruginea* means no tight grouping is evident for the sample of this species. In order to adequately characterise *P.ferruginea* using multivariate techniques a larger sample is needed. Owing to the rarity of this species, and a collectors re-

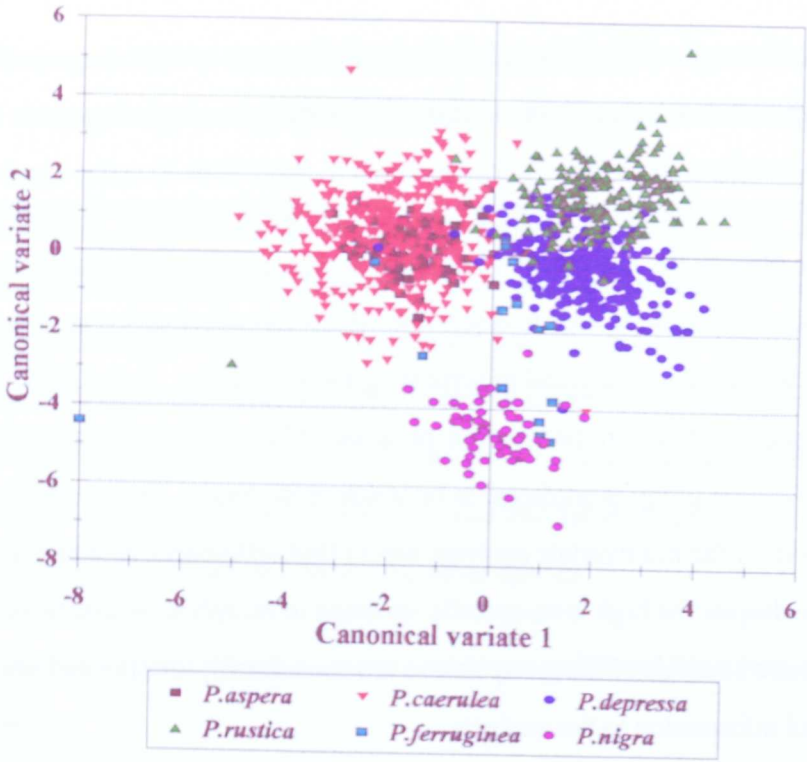


Figure 6.8a - Plot of canonical variate 1 against canonical variate 2 for samples of the six species of *Patella*. The data are separated by species, and individual scores plotted. Radula length is included in the analysis.

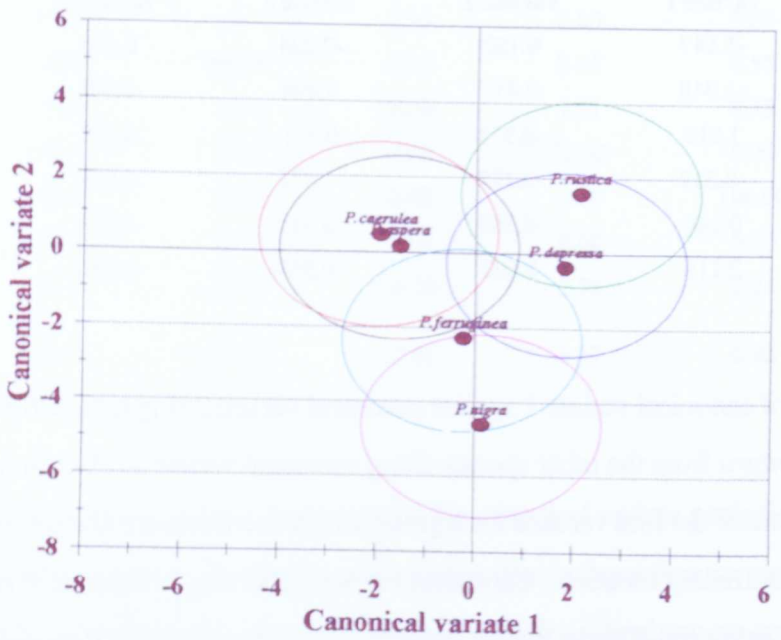


Figure 6.8b - Plot of canonical variate 1 against canonical variate 2 for samples of the six species of *Patella*. The data are separated by species, with group centroids and 95%confidence intervals of the populations shown. Radula length is included in the analysis.

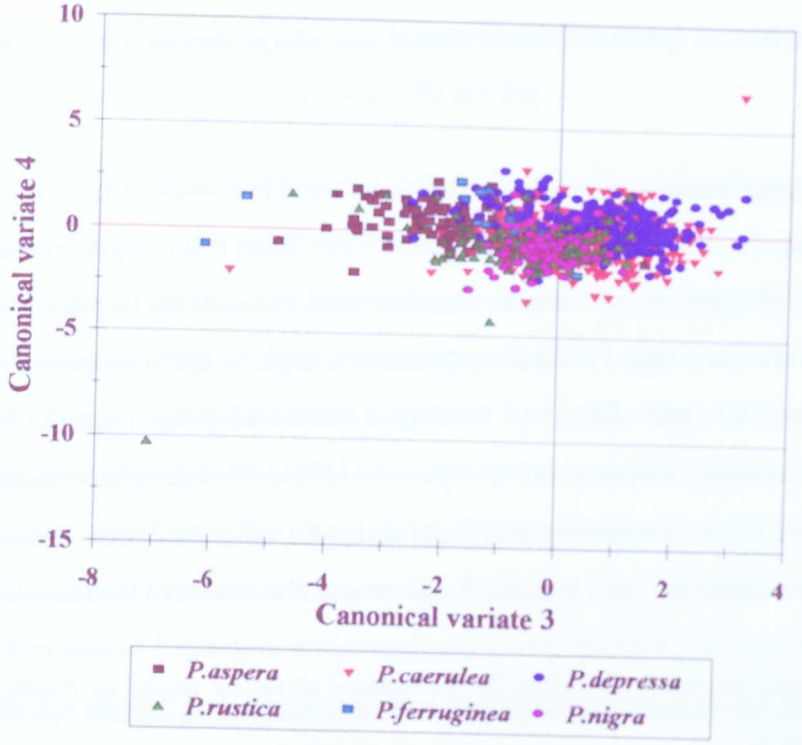


Figure 6.9a - Plot of canonical variate 3 against canonical variate 4 for samples of the six species of *Patella*. The data are separated by species, and individual scores plotted. Radula length is included in the analysis.

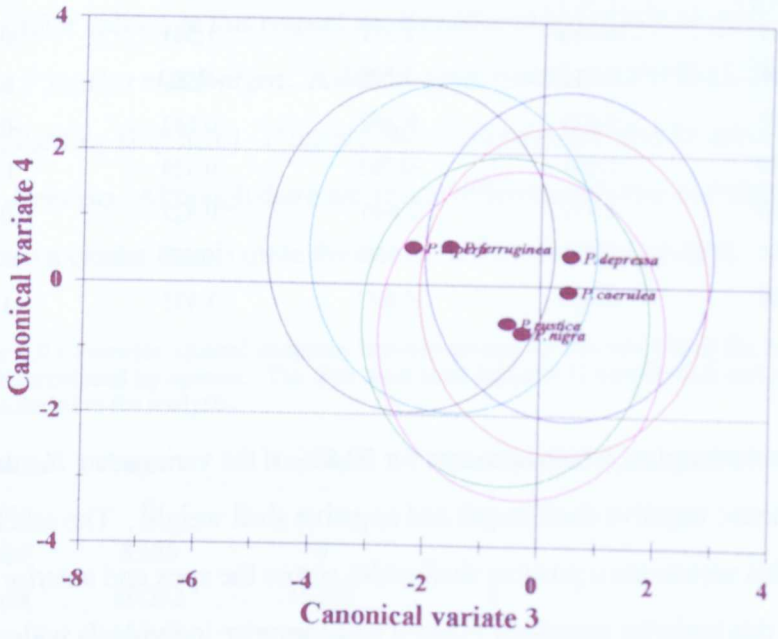


Figure 6.9b - Plot of canonical variate 3 against canonical variate 4 for samples of the six species of *Patella*. The data are separated by species, with group centroids and 95%confidence intervals of the populations shown. Radula length is included in the analysis.

sponsibility not to eradicate a species from any sample site, a large sample was difficult to obtain.

The first canonical variate which separates *P.aspera* and *P.caerulea* from *P.depressa* and *P.rustica* accounts for 51.8% of the data set (Table 6.8). There is no single dominant element amongst the coefficients for this variate. The four main elements are two positive elements (radula length and shell height (widthways)) and two negative elements (width across the shell apex and anterior to apex distance). The higher scores and group means for *P.depressa* and *P.rustica* samples compared with *P.aspera* and *P.caerulea* samples indicate that *P.depressa* and *P.rustica* have a relatively longer radula length, taller shell, more anteriar shell apex and are narrower across the apex than are *P.aspera* and *P.caerulea* of the same size.

Table 6.8 - Standardized canonical coefficients for the samples of the six species of *Patella* , samples differentiated by species. The data have been $\log_{10}(x+1)$ transformed and standardized. Radula length is included in the analysis.

Measured character	Canonical variate 1	Canonical variate 2	Canonical variate 3	Canonical variate 4	Canonical variate 5
Percentage of variance	51.8	32.8	10.9	2.9	1.5
Length	0.320	-3.014	-1.765	-5.287	0.637
Width (max)	-0.167	0.266	1.311	1.541	-0.189
Height (width)	1.229	0.016	-1.530	-0.023	1.525
Height (length)	0.189	0.425	0.260	0.142	-0.798
Apex	-1.039	1.901	-0.761	0.329	1.566
Width (apex)	-1.398	1.977	2.646	0.422	-0.572
Radula length	1.448	0.771	0.959	-0.486	0.334
Cube root of weight	-0.026	-2.835	-0.677	3.423	-1.458

The second canonical variate which accounts for 32.8% of the variance in the data set has two dominant elements: negative shell length and negative shell weight . The other elements contributing to this variate are a positive shell width across the apex and anterior to apex distance elements. On this basis the sample of *P.nigra* will comprise individuals with: a heavier shell, a greater shell length, a more anteriar shell apex and a smaller width across the apex than will individuals of the other species. *P.ferruginea* also has a relatively low group mean along the second canonical axis (Fig 6.8b) and thus shows similar differences in shape is *P.ni-*

gra compared with the other species. *P.aspera* and *P.caerulea* show almost no differences along the first two canonical axes (Fig 6.8a &6.8b).

The plots of canonical variate 3 against canonical variate 4 (Fig 6.9a & 6.9b) show that it is canonical variate 3 that separates *P.caerulea* from *P.aspera*, with *P.aspera* and *P.ferruginea* separated from *P.caerulea* and *P.depressa*. Canonical variate 4 shows few differences between the groups, although there is some separation of *P.aspera* and *P.ferruginea* from *P.rustica* and *P.nigra*. Canonical variate 3 accounts for 10.9% of the variance in the data set, it has no single dominant element (Table 6.8). The strongest element is the width across the apex measure also contributing are a negative shell length element; a negative shell height (lengthways) element and positive maximum shell width and radula length elements. This indicates that individuals of *P.caerulea* and *P.depressa* tend to be wider, particularly across the shell apex; have a smaller shell length and shell height and a slightly longer radula than individuals of *P.aspera* and *P.ferruginea* of the same size.

The pairwise squared distances in canonical space between the species show how similar species are to each other in terms of shell shape and radula length (Table 6.9). The two most similar species in this respect are *P.rustica* and *P.depressa* (6.274) and the two most distant are *P.rustica* and *P.nigra*. A dendrogram based on a UPGMA cluster analysis illustrates the similarities (Fig 6.10). *P.aspera* and *P.caerulea* are clearly quite similar as are *P.rustica* and *P.depressa*. Although there are strong differences between *P.ferruginea* and *P.nigra* the two form a cluster than is quite distinct from the other four species.

Table 6.9 - Pairwise squared distances between groups for the samples of the six species of *Patella* , samples differentiated by species. The data have been $\log_{10}(x+1)$ transformed and standardized. Radula length is included in the analysis.

From SITE	*P.aspera*	*P.caerulea*	*P.depressa*	*P.ferruginea*	*P.nigra*	*P.rustica*
P.aspera	0					
P.caerulea	8.188	0				
P.depressa	19.262	15.151	0			
P.ferruginea	14.957	20.250	17.875	0		
P.nigra	30.947	31.087	22.881	15.073	0	
P.rustica	19.674	19.199	6.274	27.988	42.634	0

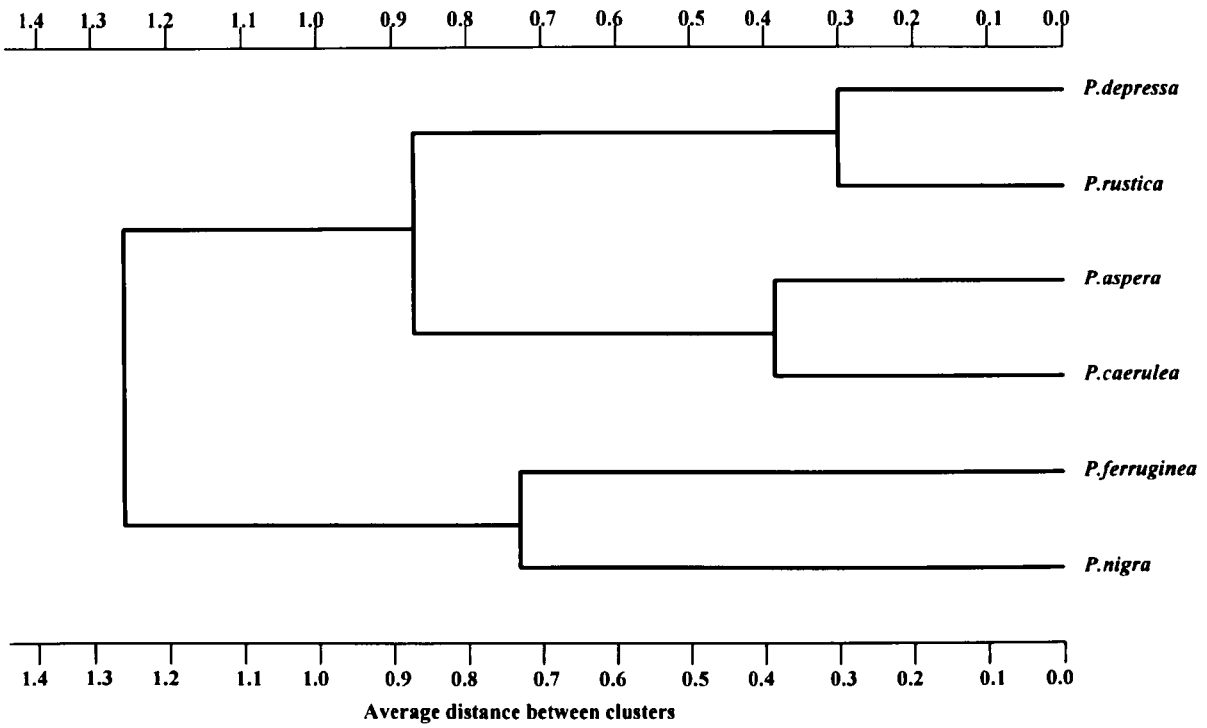


Figure 6.10 - Dendrogram based on an UPGMA cluster analysis of the pairwise squared canonical distances between the samples of the six species of *Patella*. Radula length is included in the analysis.

Table 6.10 - Pairwise squared distances between groups for the samples of the six species of *Patella*., samples differentiated by species. The data have been $\log_{10}(x+1)$ transformed and standardized. Radula length is not included in the analysis.

From SITE	*P.aspera*	*P.caerulea*	*P.depressa*	*P.ferruginea*	*P.nigra*	*P.rustica*
P.aspera	0					
P.caerulea	6.710	0				
P.depressa	8.511	10.895	0			
P.ferruginea	14.496	19.962	11.115	0		
P.nigra	30.616	30.676	15.571	15.062	0	
P.rustica	4.184	11.800	5.842	17.379	31.340	0

If the canonical discriminant analysis is repeated on the data set excluding radula length, then differences purely in shell size and shape can be investigated. A dendrogram based on such an analysis using a UPGMA cluster analysis shows the change in degree of similarity between species that results from excluding radula length (Fig 6.11). The two most similar species based on this analysis are *P.aspera* and *P.rustica* (Table 6.10), a marked change from the first analysis. Looking at the two dendrograms together (Fig 6.10 & 6.11) the differences in clustering between the two are as a result of the exclusion of radula length. Clearly *P.aspera* and *P.rustica* have similar shell shapes but markedly different relative radula lengths. Whilst

P.caerulea and *P.aspera* have similar relative radula lengths but differences in shape that are masked by the first analysis. *P.depressa* and *P.rustica* which were the two most similar species in the first analysis have been separated by the exclusion of radula length. *P.caerulea* now forms a separate group on its own, quite distinct from *P.aspera*, *P.depressa* and *P.rustica*. The relationship between *P.ferruginea* and *P.nigra* and the four other species has not changed, although the distance between the two species is increased slightly by the exclusion of radula

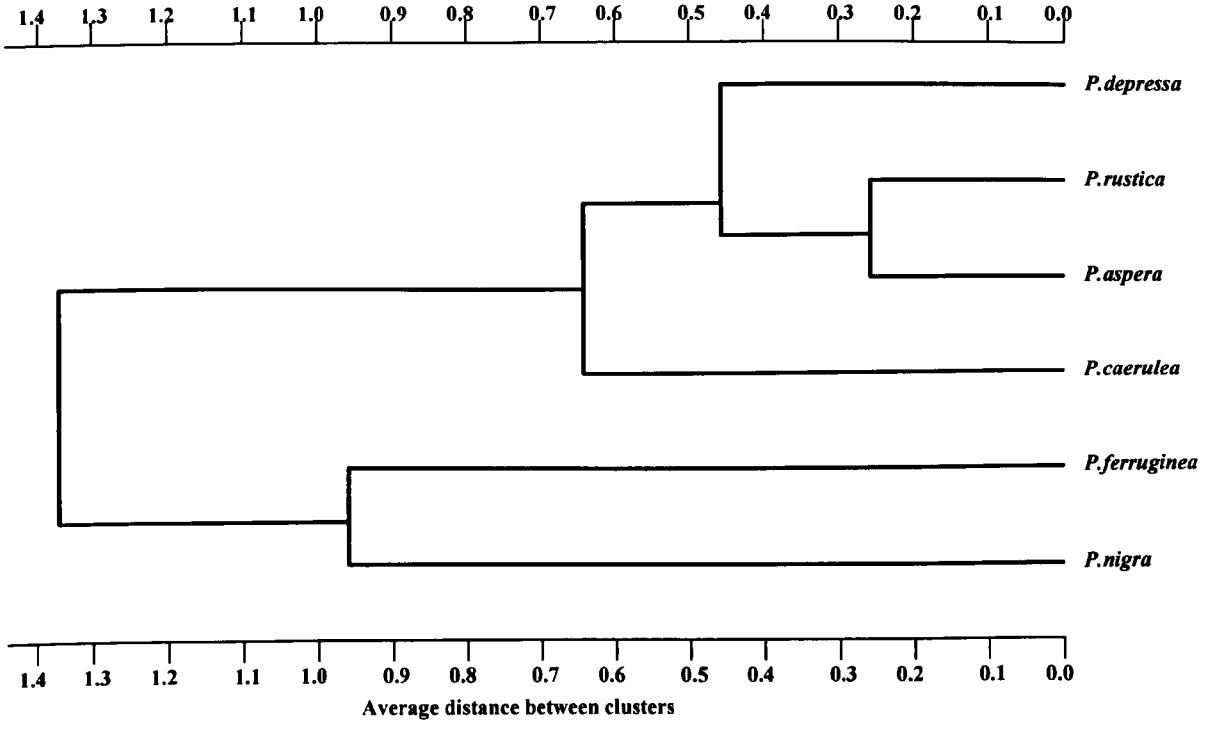


Figure 6.11 - Dendrogram based on an UPGMA cluster analysis of the pairwise squared canonical distances between the samples of the six species of *Patella*. Radula length is not included in the analysis.

length.

Table 6.11 - Linear discriminant function using cross-validation showing percentage of individuals from the six species of *Patella* samples correctly assigned to their original groups (bold). The data are differentiated by site. Radula length is not included in the analysis.

From SITE	*P.aspera*	*P.caerulea*	*P.depressa*	*P.ferruginea*	*P.nigra*	*P.rustica*
P.aspera	81.93	7.23	0.00	0.00	0.00	10.84
P.caerulea	8.59	**82.83**	2.77	2.22	0.55	3.05
P.depressa	6.79	2.26	**80.75**	1.89	1.51	6.79
P.ferruginea	6.67	6.67	0.00	**80.00**	0.00	6.67
P.nigra	0.00	0.00	0.00	0.00	**100.00**	0.00
P.rustica	15.34	0.00	3.07	1.23	0.00	**80.37**
% allocated to group	15.01	32.74	24.03	2.83	7.56	17.84
% of data set	8.71	37.89	27.81	1.57	6.92	17.10

The results of the linear discriminant function analysis (Table 6.11) generally show a lower percentage of individuals correctly assigned to their original groups than was found in the first analysis. The percentage of *P.nigra* remains unchanged at 100%, the percentage for *P.ferruginea* has gone up from 73% to 80% but the percentages for the other four species have dropped by between 8 and 13%. These results are in keeping with those of the previous chapters, where a linear discriminant function analysis carried out without radula length included as one of the variables tended to show a lower percentage of correct assignment than where all the variables were used. The results of this analysis are still instructive as they provide separation of species that show high similarities in the first analysis, that is *P.depressa* with *P.rustica* and *P.aspera* with *P.caerulea*. It is the separation along the canonical axes of these two pairs of species that will be looked at in this analysis where radula length has been excluded to see which shell characters are providing this separation.

The changes in the degree of similarity between the species are reflected in the separation of the samples along the first two canonical variate axes (Fig 6.12a & 6.12b). *P.depressa* and *P.rustica* which were most similar in terms of shell shape and radula length (from the first analysis) are partially separated by canonical variate 1 and strongly separated by canonical variate 2. In the first analysis *P.aspera* and *P.caerulea* showed virtually no separation along the first two canonical variate axes. *P.aspera* is now separated from *P.caerulea* along both of the first two canonical variates (Fig 6.12b), principally along canonical variate 2. The two pairs of

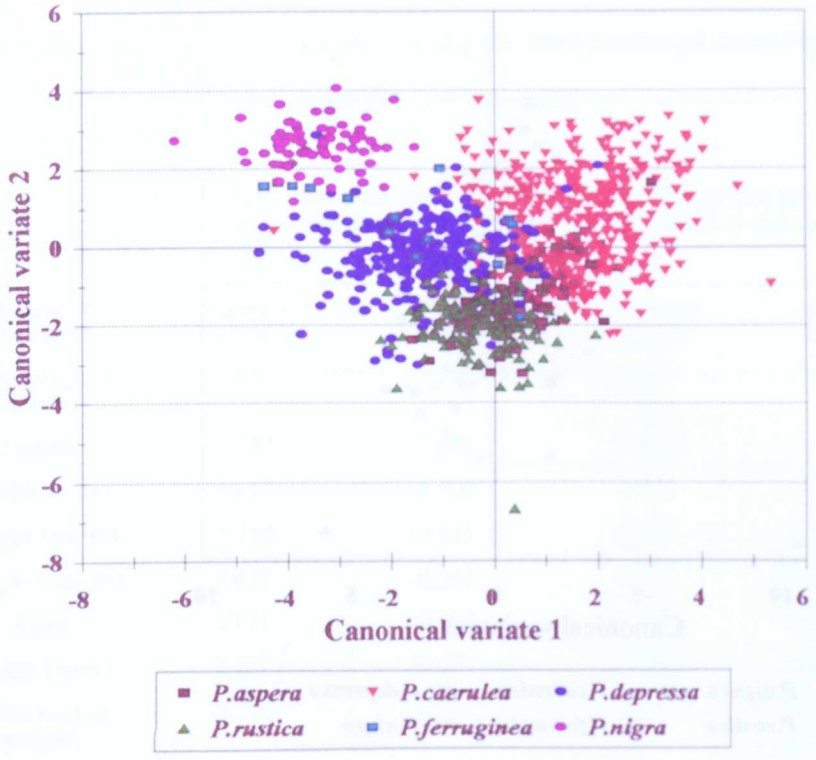


Figure 6.12a - Plot of canonical variate 1 against canonical variate 2 for samples of the six species of *Patella*. The data are separated by species, and individual scores plotted. Radula length is not included in the analysis.

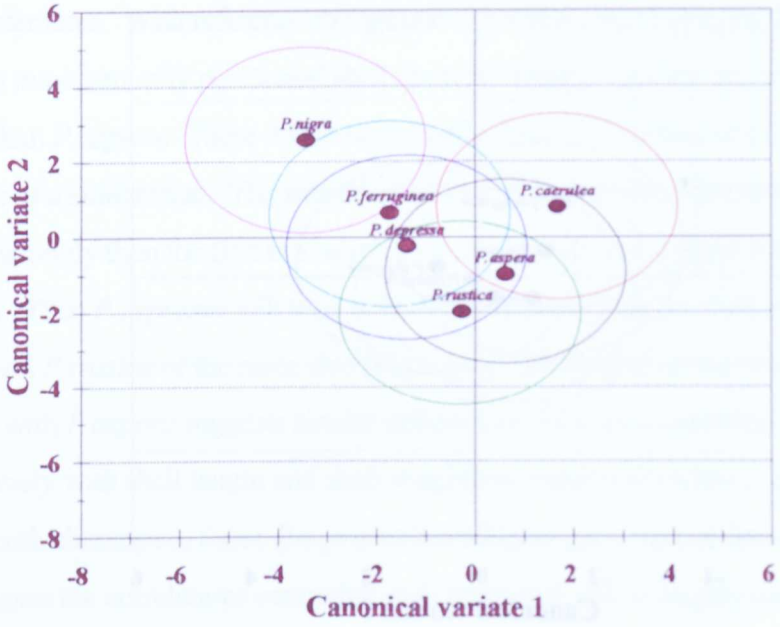


Figure 6.12b - Plot of canonical variate 1 against canonical variate 2 for samples of the six species of *Patella*. The data are separated by species, with group centroids and 95% confidence intervals of the populations shown. Radula length is not included in the analysis.

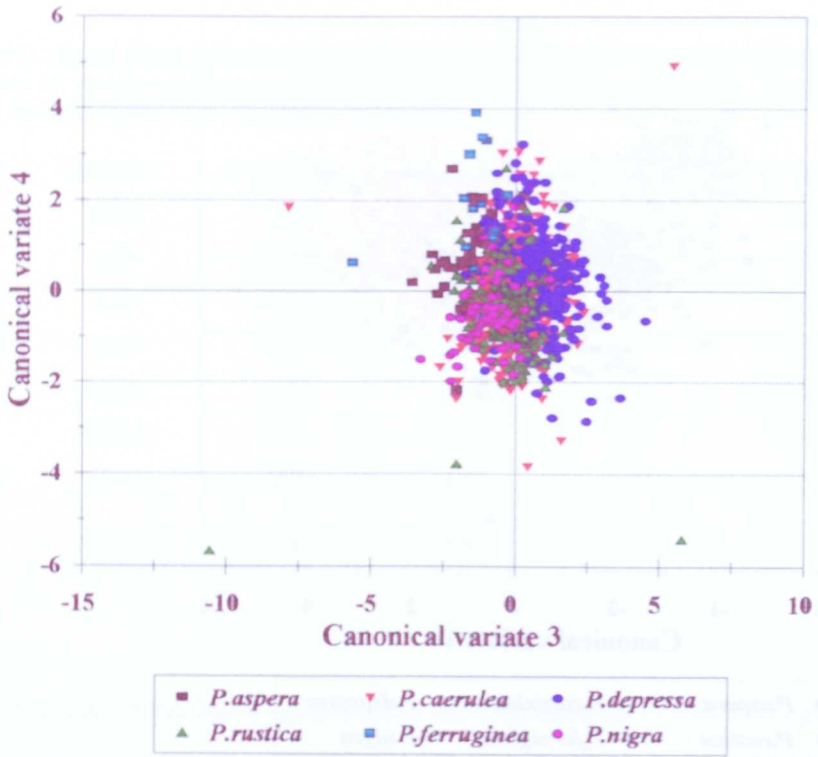


Figure 6.13a - Plot of canonical variate 3 against canonical variate 4 for samples of the six species of *Patella*. The data are separated by species, and individual scores plotted. Radula length is not included in the analysis.

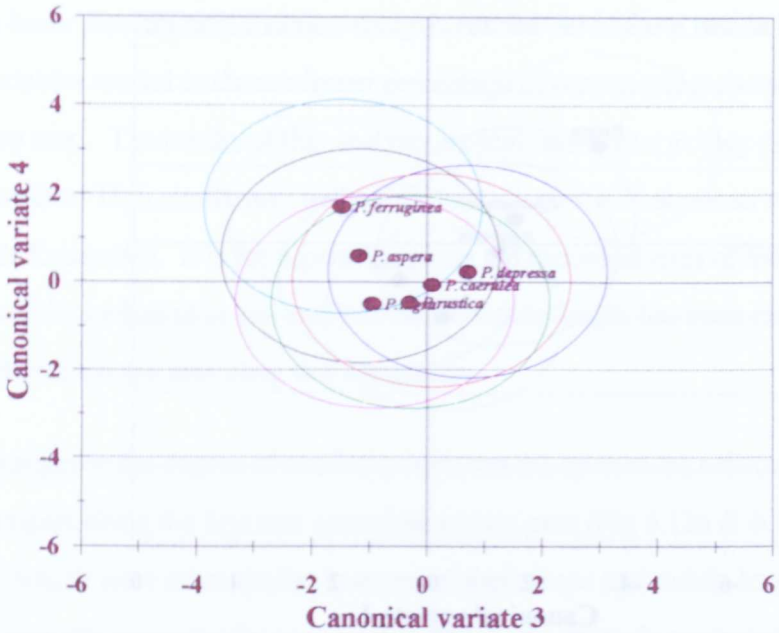


Figure 6.13b - Plot of canonical variate 3 against canonical variate 4 for samples of the six species of *Patella*. The data are separated by species, with group centroids and 95% confidence intervals of the populations shown. Radula length is not included in the analysis.

species also show some separation along the third canonical axis (Fig 6.13a & 6.13b), but this separation is not a strong as for the first two axes.

Table 6.12 - Standardized canonical coefficients for the samples of the six species of *Patella* samples are differentiated by species. The data have been $\log_{10}(x+1)$ transformed and standardized. Radula length is not included in the analysis.

Measured character	Canonical variate 1	Canonical variate 2	Canonical variate 3	Canonical variate 4	Canonical variate 5
Percentage of variance	54.5	32.2	8.4	3.2	1.8
Length	-2.283	1.692	-3.820	-3.803	1.606
Width (max)	0.413	0.360	1.717	0.932	-0.317
Height (width)	-1.190	-1.641	-0.507	0.204	1.894
Height (length)	0.057	-0.569	0.954	-0.667	-0.224
Apex	2.171	-0.556	-1.450	1.696	0.417
Width (apex)	2.467	0.109	3.212	-1.178	0.727
Cube root of weight	-2.126	1.311	0.042	3.323	-3.289

The first canonical variate is a combination of a number of characters (Table 6.12): negative shell length and shell weight and positive anterior to apex distance and width across the apex elements. With reference to Figure 6.12a and 6.12b *P.caerulea* individuals will tend to have a more centrally positioned shell apex, be wider across the apex and have a shorter lighter shell than *P.aspera*. These differences are also true of *P.rustica* when compared with *P.depressa* of a similar size. The second canonical variate which separates the two pairs of species more strongly than the first is a contrast of shell length and weight with shell height (widthways). Thus *P.depressa* will tend to have a longer and heavier shell and a smaller shell height than will *P.rustica* of the same size (Fig 6.12b). The higher group mean for *P.caerulea* compared with *P.aspera* suggests similar differences. However canonical variate 1 is correlated negatively with shell length and shell weight and canonical variate 2 is correlated positively with both characters. Since *P.caerulea* has a higher group mean than *P.aspera* along both these axes the correlations contradict each other and will be largely cancelled out. As the shell height (widthways) element is negatively correlated with both canonical variates it is this character that mainly acts to separate the two species, with *P.aspera* having a taller shell than *P.caerulea* of the same size. The width across the apex measure is a strong positive element in the first canonical variate and a weak positive element in the second canonical variate, thus *P.aspera* will tend to be narrower across the apex than will *P.caerulea*. The anterior to apex

distance measure is a strong positive element in canonical variate 1 and a weak negative element in canonical variate 2. Therefore this element will still be important in differentiating *P.aspera* from *P.caerulea*, with *P.aspera* having a shell apex closer to the anterior of the shell.

Table 6.13 - Group means of the first five canonical variates for the samples of the six species of *Patella* samples are differentiated by species. The data have been $\log_{10}(x+1)$ transformed and standardized. Radula length is not included in the analysis.

SITE	Canonical variate 1	Canonical variate 2	Canonical variate 3	Canonical variate 4	Canonical variate 5
P.aspera	0.617	-0.986	-1.177	0.587	-0.546
P.caerulea	1.684	0.792	0.102	-0.065	0.046
P.depressa	-1.372	-0.238	0.732	0.223	-0.077
P.ferruginea	-1.724	0.653	-1.480	1.704	1.688
P.nigra	-3.427	2.593	-0.941	-0.506	-0.106
P.rustica	-0.268	-1.974	-0.299	-0.469	0.189

6.4 - Discussion

6.4.1 - Field identification of the six species of Patella in the Mediterranean.

P.nigra is clearly very different from the other five species with respect to morphology. It is the only species that has a V-shaped radula. It has a well developed rachidian tooth. The shell is elongated, narrow across the apex and with the apex positioned well towards the anterior of the shell. The distinctive black head and foot and yellow pallial tentacles coupled with the shell morphology make this species readily identifiable in the field.

P.ferruginea is equally easy to identify, The heavily ribbed shell with crenulated shell margin is sufficient to separate this species from the other five. It also shares with *P.rustica* a radula with horizontally aligned teeth and a small central rachidian tooth.

P.rustica is the only one of the six species to have transparent pallial tentacles. This feature alone is diagnostic. Additionally it has radial ribs in the shell exterior with black nodules along the ribs, the longest radula length of the species, relative to shell size and as stated above it as a small rachidian tooth.

Positive field identification of the remaining species is more problematic. There do not appear to be any combination of soft body colorations that uniquely identify any of these three

species. On the basis of shell morphology a linear discriminant function allocation rule correctly assigned samples of these three species between 80 and 95% of the time. This clearly indicates that there are differences in shell morphology between the three species. However, these differences in morphology are not discrete and thus cannot be readily turned into descriptions of shell morphology that would enable positive field identification.

A different approach could be used, that is the linear discriminant function derived from the analysis can be used in the identification of individuals collected at a later date. The original data were used as a training set (Krzanowski 1988) to derive an allocation rule by which the six species can be separated. If the same shell measurements as used in the analysis are taken from individuals that need identifying, then these measurements can be submitted to the allocation rule to determine the species of the individual. The rule would probably be correct more than 80% of the time (see Section 6.3.5). The accuracy of the allocation rule could be checked by using spiked data, where the identity of each individual in the spiked sample was checked using electrophoresis.

The *P.aspera* sample used in this chapter was relatively small and both *P.aspera* and *P.depressa* were only collected from a couple of locations. The use of a linear discriminant function could be strengthened by including more data for these two species in the training set. This would not necessarily increase the percentage correctly assigned by the rule but would provide more realistic allocation percentages for each species based on the shell measures used. The linear discriminant function would in all probability have a lower chance of correctly allocating individuals, this being because larger samples and samples from different sites will probably realise more of the scope for variation that the species possess.

6.4.2 - P.nigra and P.ferruginea

P.nigra appears to be very different from the other Mediterranean species. The V-shaped radula with prominent racidian tooth is more like those found in South African limpets (Koch 1949) than those of the Mediterranean. It is also the only one of the Mediterranean species that displays territorial and gardening behaviour (Frenkiel and Mouëza 1982); again such behaviour is found in a number of species of South African limpet (see Branch 1981 for a review).

Its distribution appears to be consistent with a species that has spread up from Southern Africa and is at the northern limit of its distribution in the Mediterranean. It is found along the west coast of Africa as far south as Angola and Namibia (Hodgson *pers. comm.*). It extends into the Mediterranean predominantly along the North African coast. It is found as far west along this coast as Alger (Frenkiel and Mouëza 1977). It has been reported along the Spanish Mediterranean coast at Malaga (Grandfils and Vega 1984) and as far west as Estopona (Hawkins *per. comm.*) although it is rare on the north coast of the Mediterranean. Such a distribution is consistent with a species that has spread into the Mediterranean from the west coast of Africa (Pérès 1967).

P.ferruginea is generally regarded as a relic species endemic to the islands in the western Mediterranean basin (Fischer-Piette 1935, 1948; Christiaens 1973; Frenkiel and Mouëza 1977, 1982). It has also been recorded on mainland coasts, for example Tuscany-Italy (Biagi and Poli 1986) and Algeria (Pallary 1900; Frenkiel and Mouëza 1982). The island populations of this species tend to be more dense and have a greater proportion of large individuals than the mainland populations which appear to have a greater proportion of juveniles (Frenkiel and Mouëza 1982; Biagi and Poli 1986)

The rarity of this species is worth comment. It would appear that its distribution is not limited by competition with other patellids as in ecological studies in Algeria it would seem that *P.ferruginea* limits the distribution of other species (*P.nigra*, *P.rustica* and *P.caerulea*) rather than the other way around (Frenkiel and Mouëza 1982). It is possible that this species is more susceptible to pollution than other some other species and that this increases its rarity. Aversand (1986) in artificial transfer experiments suggests that such transfers into suitable habitat are only successful in areas of low pollution. Additionally *P.ferruginea* grows to a large size and thus would probably have suffered more at the hands of people collecting limpets for food or bait.

The ecology of this species needs considerable further investigation. The reports on ecological habit in the literature are contradictory, either that or the species displays vastly different distribution patterns in different areas (contrast Biagi and Poli 1986 with Frenkiel and Mouëza 1982). Biagi and Poli say that in Tuscany the species is almost exclusively found on open rock in the supralittoral (the very top of the shore) and is found covered in the lichen *Verrucaria*

symbalana. They relate that *P.ferruginea* appeared not to be very active compared with *P.rustica* in the same zone - indeed they never found any *P.ferruginea* grazing, found no evidence of grazing and only on one occasion did they find an individual not clamped down hard on the rock. They give a similar vertical distribution for *P.ferruginea* from Corsica. Frenkiel and Mouëza (1982) regard *P.ferruginea* as a mesolittoral limpet found above the red algal turf zone and in the area of maximum wetting by wave action at the same level as *P.caerulea.*

In this study *P.ferruginea* was only found at two locations, Calvi and Isle Rousse, both in Corsica. At both locations *P.ferruginea* was not found on the breakwaters in calm weather either in the mesolittoral or the infralittoral zone. Despite extensive searching in the upper mesolittoral and the supralittoral it was not evident in these zones. In rough weather it was found grazing in the mesolittoral, just above the red algal fringe. Its apparent absence during calm weather tends to suggest it makes use of retreats such as cracks or crevices in the lower mesolittoral and comes out to graze during periods of high wave action. It has been found to exploit such habitats on natural shores (Hawkins *pers. comm.*). Although the zone occupied was found to be different from Biagi and Poli (1986), it may explain why they did not find it grazing, grazing only occurring during periods of high wave action. Clearly work on the ecology of this species would benefit our understanding of it. Additionally if it is indeed sensitive to pollution then its role as an indicator organism could be accessed.

A final point of interest concerning *P.ferruginea* is that of the 15 adult *P.ferruginea* collected, two had juveniles on the top of the shell, one juvenile having created a clearly visible home scar (these were the only two *P.ferruginea* found less than 1cm long). This has also been reported by Aversand (1986). The possible role of the adult shells as a nursery for juveniles would stand further investigation. The possibility exists that the species has dwarf males as is the case with some South African limpets (Branch 1981).

The accurate field identification of limpets is important if ecological investigations or experiments are being carried out. With the grazing effects of this group important in the distribution of rocky shore algae, the ability to distinguish the species is essential if the roles of each of the species are to be separated from one another.

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7 - General Discussion

7.1 - Intra-specific genetic variation

High levels of gene flow were inferred between mesolittoral and infralittoral samples of *P.caerulea* at the locations studied. This would appear to be consistent with the mesolittoral and infralittoral morphotypes constituting a single gene pool. Similar results have been found for other bimodally distributed limpets: *P.aspera* (Côrte-Real *et al.* 1992) and the Antarctic limpet, *Nacella concinna* (Beaumont and Wei 1991). In both cases samples of the two morphotypes were markedly different in shape, with the low shore morph having a flatter broader shell, yet in neither study were significant genetic differences found between samples of the two morphotypes.

The levels of gene flow inferred from the genetic identities (I) for samples of *P.caerulea* taken from the same island were high and consistent with those found in other work on gene flow in *P.caerulea* (Sella *et al.* 1985; Badino *et al.* 1986). Such high levels of gene flow are consistent with a species that has a planktonic larval phase (Gooch 1975; Flowerdew 1984; Crisp 1978; Furman 1990). The levels of gene flow between the regions for both *P.caerulea* and *P.depressa* are relatively low, when compared with work on *P.caerulea* (Sella *et al.* 1985; Badino *et al.* 1986), *Nacella concinna* (Beaumont and Wei 1991), littorinids (Johannesson and Johannesson 1990; Ward 1990) and bivalves (Beaumont and Khamdan 1991; Koehn 1991; Gardner 1992): they are, however, in agreement with the most recent work on the genetic differences over a large geographic range in patellids (Côrte-Real 1992).

The length of the larval stage of patellids is in the order of 10 days in the laboratory (Dodd 1957), and from this one might expect high levels of gene flow between regions (Gooch 1975; Crisp 1978). However, the length of larval stage in the laboratory has not been confirmed in a natural situation and the actual time spent in the plankton is often less than that found in the laboratory (Prince *et al.* 1987, 1988; Mc Shane *et al.* 1988). The length of time spent in the plankton does not relate in a simple way to the distance dispersed and can be modified by a number of hydrographical and biological factors see (Hedgecock 1986; Furman 1990; Pedrotti and Fenaux 1992). Thus in order for the reason for the apparently low gene

flow between regions to be ascertained, studies on the larval life history, larval behaviour and realised dispersal distances need to be carried out (see Section 7.4.2).

7.2 - Intra-specific variation in shell shape and its implications

With respect to shell shape, one fact consistently reasserts itself, patellids show a very high level of intra-specific variation (Moore 1934; Comfort 1946; Brian and Owen 1952; Christiaens 1965; Davies 1969; Bacci and Sella 1970; Sella and Bacci 1971; Bannister 1975; Sella 1976; Bowman 1981; Baxter 1982, 1983; Côrte-Real 1992). This is borne out by this study. Indeed, it is the main reason for the profusion of described 'species' and varieties in the early literature (see Christiaens 1973; Powell 1973).

The high genetic identity between samples of *P.caerulea* from the mesolittoral and infralittoral shore zones within a site (see Chapter 3) and yet the obvious differences in shell shape between these same samples, suggest that this intra-specific variation in shape is environmentally induced. In work on other bimodally distributed limpet species, similar differences in shell shape have been attributed to phenotypic plasticity (Beaumont and Wei 1991; Côrte-Real *et al.* 1992). Transplant experiments in *Patella* showing a change in growth pattern (Moore 1934) coupled with the lack of apparent genetic differentiation between morphs (Beaumont and Wei 1991; Côrte-Real *et al.* 1992) would seem to support such a view. The basis of phenotypic variation has been shown to have both genetic and environmental components (e.g. Cook 1965, 1967; Newkirk 1975; Lam 1989; Boulding & Hay 1993) and selection for shell form has been shown in the field (Kitching 1966; Johannesson 1986; Cain 1988a, 1988b; Byers 1990)

Whether the variation in shape is due to phenotypic plasticity or selection of advantageous genotypes (and it is probably a combination of the two), it is still the environment that is the controlling factor.

It would appear that in limpets different shell shapes are advantageous: at different shore levels (Moore 1934; Davies 1969; Rao and Ganapati 1971; Sella 1976); at different levels of exposure to wave action (Branch and Marsh 1978) and when preyed on by different species (Hockey and Branch 1983; Lowell 1986, 1987). It follows that a species with a wide scope for variation in shell shape will potentially be able to exploit a wider variety of habitats. It

would appear that rather than physical factors, the distribution of species of *Patella* is often restricted by the presence of other patellids (Branch 1981, 1984; Frenkiel and Mouëza 1982). This would appear to be the case in *P.caerulea* where it has a bimodal distribution on some shores being found in the mesolittoral and the infralittoral zones, but is virtually excluded from the infralittoral fringe by *P.aspera* (see Chapter 3).

The high intra-specific variation in shape presents a problem when investigating morphological differences between species. Where the samples are allopatric, one cannot determine the extent to which the observed differences in shape are simply products of different environments. Thus, one cannot predict whether the differences found will hold true should the species be found sympatrically. This point emphasises the need to use another technique such as electrophoresis to reinforce the morphological analysis.

7.3 - The salinity crisis, glaciation, speciation and distribution.

The salinity crisis of the late Miocene and subsequent glacial events in the Pliocene and Quaternary probably played a vital role in speciation of patellids and their present day distribution.

The genetic identity between the congenerics *P.caerulea* and *P.depressa* was found to be 0.48 (see Chapter 5), using Nei's estimate of divergence (Nei 1972) this gives an approximate time of divergence of about 4 million years (my) ago. That is *P.caerulea* and *P.depressa* probably diverged from a common root at about this time.

In the late Miocene (35-10 my ago) the junction of Europe and North Africa and the formation of the Gibraltar Sill separated the western Tethys sea from the rest of the Tethys to form what was to become the Mediterranean (Berggren 1969; Berggren 1972; Berggren and Hollister 1974). In the late Miocene, about 5.5 my ago the Western Tethys experienced a drastic drop in level that resulted in what is commonly known as the 'Salinity Crisis' (Berggren and Hollister 1974; Hsü *et al.* 1977). This desiccation of the western Tethys resulted in the disappearance of the majority of the Miocene fauna from this area (Hsü *et al.* 1977), with the end result that the present day fauna is similar to that found during the Pliocene, but very different to that from the Miocene.

Approximately 5 my ago, in the early Pliocene, there was an Atlantic water transgression flooding the old western Tethys and establishing the Mediterranean as we know it today (Berggren and Hollister 1974). This transgression allowed a large scale invasion of warm water Atlantic fauna into the Mediterranean (Blanc 1968) in what was at the time a region of low species diversity (Berggren and Hollister 1974).

During the Upper Pliocene and the Lower Quaternary there were a series of glacial events (see Chapter 1 Section 1.4), where the inflow into the Mediterranean was of deep water currents, with the surface current flowing out into the Atlantic. The interglacial periods were characterised by a transgressive phases where both the deep water and the surface currents reversed direction.

The divergence of *P.caerulea* and *P.depressa* from a common stock probably occurred somewhere in the time frame between the Upper Miocene and the Upper Pliocene. It is possible that the root stock of these two species was split by the Gibraltar Sill at the onset of the salinity crisis, and that the subsequent separation allowed allopatric divergence to occur such that by the time of the Upper Pliocene transgression speciation had occurred. The markedly different environmental conditions that existed between the Mediterranean and the Atlantic at this time may have helped to drive the divergence of the separated stocks. The harsh environmental conditions present in the Mediterranean at that time may have been too severe for any limpet species to survive. The other possibility that is consistent with the approximate time of divergence is that the original stock was divided by one of the regressions of the Upper Pliocene or Quaternary. The cold period could have pushed the northern limit of the original stock south to some point on the west African coast, but left a part of the stock isolated in the Mediterranean.

Whenever the divergence did occur and whatever caused it, the present day distributions of *P.caerulea* and *P.depressa* pose an interesting question. With the phases of transgression and regression during the Quaternary in the Mediterranean, what has stopped *P.depressa* from extending further into the Mediterranean or *P.caerulea* extending into the Atlantic? A number of possible reasons present themselves:

1 - The surface current patterns acted to prevent the possible range extensions of each species. The present day surface current patterns appear to support this. There appears

to be a barrier to gene flow in the region of Cabo de Palos (Spain) that limits the extent to which *P.depressa* extends into the Mediterranean (see Dando and Southward 1981). The problem with this is that other littoral species have invaded the Mediterranean during transgressive phases (Blanc 1968).

2 - The two species appear to be adapted to very different environments. Although there may have been larval supply of *P.depressa* into the Mediterranean during transgressions and of *P.caerulea* into the Atlantic during regressions, the species were not able to establish and reproduce due to the different conditions and/or competition from the species already present (Frenkiel and Mouëza 1982).

3 - In light of point 2, if one were to have an accurate record of the distributions of the two species during the Quaternary, it may reveal an overlap in the ranges of the two species the geographic boundary of which shifts dependent upon whether there was a transgression or a regression at the time. In a regression *P.caerulea* may extend its range out of the Mediterranean and during a transgression (which is the current phase) *P.depressa* extends into the Mediterranean.

7.4 - Further work

Possible further work on Mediterranean patellids can be divided into two categories: work directly relating to results from this study, answering specific questions that have arisen and more general avenues of research that would help understand the speciation and present day distribution of patellids in the Mediterranean and on a broader geographic scale.

7.4.1 - Specific work

7.4.1.1 - Tagging experiments

Investigation into the possible migration of mesolittoral *P.caerulea* into the infralittoral (see Chapter 3 Section 3.4). In Chapter 3 it was hypothesised that the infralittoral populations of *P.caerulea* were formed by the migration of mesolittoral *P.caerulea* , above a certain shell size, through the infralittoral fringe into the infralittoral, establishing and maintaining these infralittoral populations. Further, it was suggested that it may be only those mesolittoral individu-

als that have a subplanar shell shape that migrate or that it is predominantly individuals of this shell shape that survive having migrated to the infralittoral zone.

Tagging experiments, marking mesolittoral limpets, with for example micromarkers (C. Nobles unpublished), would help test these hypotheses on the origin of the infralittoral populations of *P.caerulea*. Such marking experiments have been carried out sucessfully to examine aggragation of *P.vulgata* under fucoids in the Isle of Man (C. Nobles unpublished). The markers of this type can last more than two years in the field (C. Nobles *pers. obs.*). Such tagging experiments could be extended to investigate the exclusion of one species of *Patella* by another (Frenkiel and Mouëza 1982), as appears to be the case with *P.aspera* excluding *P.caerulea* from the infralittoral fringe in the Mediterranean. Tagging a number of mesolittoral *P.caerulea* and then clearing (and keeping clear) *P.aspera* from the infralittoral fringe would determine whether it is the presence of *P.aspera* that prevents *P.caerulea* from occupying this zone on the shore in any number.

7.4.1.2 - The boundary and overlap between *P.caerulea* and *P.depressa*.

Detailed investigation of the boundary and overlap in distribution between *P.caerulea* and *P.depressa* in the Spanish Mediterranean coast needs to be carried out. With the revelation by electrophoretic investigation of loci that are diagnostic between *P.caerulea* and *P.depressa* (see Chapter 5 Section 5.3.6) the two species can be distinguished to a very high level of statistical probability. In light of this the true extent of the penetration of *P.depressa* into the Mediterranean could be investigated by the collection and electrophoretic analysis of samples of *Patella* from along the Spanish Mediterranean coast. This would also allow one to determine the degree to which the ranges of the two species overlap along this coastline. If sympatric samples of the two species were found then morphometric characterisation through the application of canonical discriminant analysis could be used. This would not suffer from the potential problems, outlined earlier, of characteristing the two species on the basis of allopatric samples. It would also allow one to establish differences in shell shape and soft body characteristics that held true for sympatric samples.

7.4.1.3 - Middens and the fossil record

The multivariate characteristation of the six Mediterranean species of *Patella* allows the identification of individuals with a probability of being correct in excess of 80% of the time for any species, based on the shell measurements alone (i.e. not including the radula length). It should be noted that the probability may be marginally lower for individuals outside the training set (Krzanowski 1988). With such high probabilities it would be possible to use the derived cross-validated linear discriminant function to identify individuals found in shell middens or in the fossil record.

The power and validity of this method would be augmented by the use of sympatric samples where possible. Also, the more samples from different locations for a species used in the training set, the greater the chance of covering the majority of the scope for morphometric variation that that species possesses.

The use of patellids as a food source and their subsequent accumulation in middens (Cunliffe and Hawkins 1988) can be used to look for changes in the geographical range of species. Middens such as that found in the La Riera cave are often built up over time, and are often layered in such a way that levels can be aged. The appearance of the shell of a species in only some levels of a midden may indicate changes in the geographic distribution of that species. If the layers the species is present in can be aged, then it may be possible to relate the appearance or disappearance of that species from the region to known event in the Quaternary, such as a glacial event. The changes in relative abundance of different patellids through the levels of a midden may not, as is often suggested, reflect active selection of a different food source or a decrease in abundance due to over exploitation, but rather changes in relative abundance of the species in question in the region due to changes in geographical range.

7.4.2 - General avenues of research.

The aspect of patellid biology that is in urgent need of study, to enhance our understanding of the groups ecology, distribution and population genetics, is the larval dispersal and recruitment. There have only been two studies on larval development in *Patella* (Smith 1935; Dodd 1957) and information on this subject is very limited. Patellids show external fertilisation and yet no information exists about what range fertilisation can occur over. Can, for in-

stance, gametes released by mesolittoral individuals fuse with those from infralittoral individuals? In genetic work this is important, in order to determine what constitutes a breeding unit (Johnson and Black 1984). Similarly, the scale upon which larval dispersal occurs is important in determining what constitutes a stock (Shepherd *et al.* 1992) and yet virtually nothing is known about the potential and realised ranges of dispersal in *Patella*. Clearly where a species is rare (e.g. *P.ferruginea*) or heavily fished (e.g. *P.aspera*) then information about breeding units and stocks is essential before effective management of the species can be started.

The range of larval dispersal of a species is dependent upon a wide range of physical, chemical or biological barriers or innate behaviour mechanisms and physiological constraints. Examples include: the maximum time a larvae can spend in the plankton (Scheltema 1986); the shortest time after which it is competent to settle (Scheltema 1986); current patterns, directions and strengths (Scheltema 1971; Pedrotti and Fenaux 1992); intense predation and dilute food sources (Hines 1986); larval behaviour such as vertical migration (Wolf 1973; Cronin and Forward 1982) and temperature and salinity (Hedgecock 1982, 1986).

Laboratory investigations into the behaviour, tactic responses and settlement cues of *Patella* larvae would be an invaluable step in accessing the dispersal potential and distribution of patellids. In the laboratory the larval duration of *P.vulgata* was found to be about 10 days (Dodd 1957). More detailed investigations into the range of larval duration; the effect of temperature on this duration and larval duration in more natural conditions would be greatly beneficial. Once the duration and behaviour of the larvae is better understood, sampling of the plankton (Scheltema *et al.* 1989; Pedrotti and Fenaux 1992) and manipulative field experiments (Prince *et al.* 1987, 1988; Shepherd *et al.* 1992) coupled with information about local and regional current patterns could be used to assess the realised range over which larval dispersal occurs.

Another important reason to investigate the larvae of *Patella* is the heterozygote deficiencies found in the adult populations. Work on mussels has lent weight to the idea that the heterozygote deficiencies found in adult populations are generated in the larvae or at early settlement (Gosling and Wilkens 1985; Mallet *et al.* 1985; Beaumont 1991; Fairbrother and Beaumont 1993) and this has been proposed as a reason for the heterozygote deficiencies found in populations of *Siphonaria jeanae* (Johnson and Black 1982, 1984a). Determining

whether the heterozygote deficiencies in adult populations of *Patella* are a result of a Wahlund effect (see Section 4.4.1), or a product of selection on larvae is essential to the understanding of the population genetics of *Patella*. The genetic analysis could be carried out by the electrophoresis of early settlers (Fairbrother and Beaumont 1993) or the larvae (Hu *et al.* 1992), however, due to the difficulty of distinguishing the different species of *Patella* as juveniles (Bowman 1981) it would be preferable to use laboratory crosses (Mallet *et al.* 1985; Beaumont 1991). Obtaining such crosses for *Patella* species would probably be difficult, yet it is information about the larvae that is needed to further our knowledge of the patellids.

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