SYSTEMATICS AND POPULATION GENETICS OF THE SOUTH AFRICAN FRESHWATER CRAB FAUNA (DECAPODA: POTAMONAUTIDAE: *Potamonautes*)

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Declaration

I, the undersigned hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part been submitted it at any university for a degree.

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Abstract

In the present study, the systematic status, aspects of the evolutionary biology and phylogenetic relationships among species of the African freshwater crab genus *Potamonautes* in South Africa are examined. Systematic research between allopatric populations of *P. brincki*, using allozyme and morphometric data has revealed the existence of a new undescribed freshwater crab species occurring in mountain streams of the Cape Peninsula. This species is described in the present study. In addition, the diagnostic value of carapace dentition patterns in the taxonomy of freshwater crabs is explored among two toothed river crab species from South Africa. The latter study utilized *P. warreni* and *P. unispinus* where considerable variation in the dentition pattern of the former species has been recorded. Fixed differences in allozyme loci demonstrated that these two taxa should indeed be considered separate, yet closely related species, while the morphometric data failed to reveal this taxonomic separation. The holotype of *P. warreni* is re-described. The relationship of hybrid taxa between *P. depressus* and *P. clarus* populations are investigated. Evident from this study is that populations that occur equidistant from the two parental taxa have undergone extensive introgressive hybridization. Considering the pronounced sequence divergence and the occurrence of fixed allozyme loci between populations, it is postulated that the hybrid populations should be considered to be on a unique evolutionary trajectory worthy of conservation. The population genetic structure of *P. calcaratus* is examined as this species is unique in its occupation of water holes. Results demonstrate that the genetic population structure is the result of recent colonization and moderate gene flow among populations. Phylogenetic relationships between the southern African freshwater crab fauna is investigated with the use of sequence data from two mitochondial genes (12 S rRNA and 16 S rRNA), allozymes and morphology in an attempt to firstly, test the usefulness of freshwater crabs as biogeographic indicators, secondly to explore

the relationship among hybrid taxa, and thirdly to examine Bott's (1955) subgeneric divisions. Results demonstrated that freshwater crabs can be used as biogeographic indicators, that hybrid taxa are phylogenetically closely related and that Bott's subgeneric divisions have no systematic basis. Among the three data sets, the sequence data provided the best resolution, while the phylogenetic inferences derived from the allozyme data and the morphology was limited.

Uitreksel

In hierdie studie word die sistematiek, aspekte van die evolusionêre biologie en filogenetiese verwantskappe tussen spesies van die varswater krap genus *Potamonautes* ondersoek in Suid Afrika. Navorsing op geografies geisoleerde populasies van *P. brincki* word met behulp van allosieme en morfometriese data ondersoek en dui daarop dat daar 'n nuwe onbeskryfde spesie op die berge van die Kaapse Skiereiland voorkom. Die nuwe spesies word beskryf in hierdie studie. Die diagnostiese waarde van tand patrone in varswater krappe word tussen twee spesies *P. warreni* en *P. unispinus* ondersoek. Genetiese data dui daarop dat alhoewel *P. warreni* morfologies baie naverwant is aan *P. unispinus,* is genoegsame verskille in allosiem lokusse wat daarop dui dat die twee taksa volwaardige spesies is. Die holotipe van *P. warreni* word herbeskryf. Die verwantkap tussen populasies van twee hibried taksa, *P. depressus* en *P. clarus,* word ondersoek. Resultate van hierdie studie dui daarop dat noemenswaardige verskille voorkom tussen die twee hibridie taxa en dat die hibriede populasies geneties verskillend is. 'n Argument word aangevoer vir die beskerming van hibriede taksa. Die populasie genetika van *P. calcaratus* word ondersoek en vergelyk met vorige studies. Die data dui daarop dat minimale geen vloei tusses populasies is en dat die populasie struktuur waarskynlik die resultaat is van onlangse kolonisasie. Die filogenetiese verwantskap tussen die suider Afrikaanse krappe word ondersoek met DNA, allosieme en morfologiese karakters met drie hoofdoele. Eerstens om die gebruik van varswater krappe as biografiese indikatore te toets. Tweedens om die verwantskap van hibriede taksa te ondersoek, en derdens om die status van Bott (1955) se subgenera te bepaal. Resultate dui daarop aan dat varswater krappe wel gebruik kan word as biografiese indikatore. Hibridieserende taxa naverwant is derdens en dat Bott se subgenera geen taksonomiese basis het nie. Die DNA data het die beste resolusie gebied vir die filogenetiese vrae, terwyl die allosieme en morfologiese data 'n laer resolusie bedied het, en meer beperk was.

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Chapter 1

1.1 General Introduction

South Africa covers approximately 1 400 000 km^2 (0.8 % of the global land surface) and contains a remarkable biological diversity estimated at between two hundred and fifty thousand to a million species (Myers, 1990; Gibbons *et al.*, 1999). The country ranks as the third-most biologically rich country in the world with the biological diversity being well distributed among the five biomes that occurs in South Africa (fynbos, forest, karoo, grassland and savanna). A prime example is the Cape Floristic Kingdom (fynbos) that contains the highest recorded species diversity of any similar-sized temperate or tropical region in the world, while the Karoo biome contains one third of the world's succulent plant species. In addition, a wealth of animal life exists throughout the country. South Africa hosts an estimated 5.8 % of the world's mammals, 8 % of the birds, 4.6 % of the reptiles, 16 % of the total number of marine fishes and 5.5 % of the described insect species. Myers *et al.,* (2000) predicts that biological hotspots, such as South Africa could contain sizeable proportions of endemic invertebrates. The patterns of diversity exhibited by the South African flora and fauna are not simple to explain and are likely to be governed by a multitude of factors of which climate, geology and ecology are amongst the most important.

Systematic studies on the floral (e.g. Goldblatt, 1978) and faunal taxa have received much attention and the diversity of most groups is well documented. However, recent research into the genetic structure of some of these "well studied" taxa has shown that the species diversity is likely to be underestimated (Crowe *et al.*, 1994). In contrast, the biodiversity of invertebrates, and more particularly aquatic, inland invertebrates

has received relatively little systematic attention. Despite considerable early taxonomic work on freshwater invertebrates, the systematics of most aquatic invertebrate groups can be regarded as "chaotic" - or at least weakly developed (Allanson *et al.,* 1990) and remains an area where considerable research effort is warranted. In most instances South Africa's freshwater biologists have based the identity of aquatic invertebrates on old taxonomic studies and many of these early references form the framework for modern research. This is especially true when considering the recent studies carried out on aquatic taxa in countries such as North America, Australia and New Zealand (Davies *et al.*, 1993). The inability of South African systematists on the whole, to identify and catalogue levels of species diversity and endemism of aquatic invertebrates suggests that we are ill equipped to gauge species losses. Although there have been repeated appeals for researchers to document exiting levels of biodiversity amongst aquatic invertebrates, few modern monographs on the South African fauna have been published.

Of particular interest is the systematic status of freshwater crustaceans of southern Africa. Major studies on the southern African freshwater crustacean fauna have been undertaken on most groups. However, the majority of these studies were conducted during the earlier part of this century. Generally, the distribution of freshwater crustaceans in southern Africa follows a pattern similar to that observed among other freshwater invertebrates (Harrison, 1978). Two main groups of freshwater crustaceans can be distinguished on the sub-continent. The first group is comprised of cold stenothermal fauna with Gondwanan affinities, while the second group has a Pan-African origin. The majority of the micro-crustaceans (such as amphipods and

isopods) belong to the first group and closely related groups are known from Australia and South America having a Gondwanan link with Africa. The macro-crustaceans (such as prawns and freshwater crabs) belong to the second group and comprise migrant crustaceans with a suspected African origin. The macro-crustaceans of southern Africa are further divided into five distinct elements: (1) widely tolerant species (2) stenothermal, tropical species (3) highveld climate species (4) mountain species and (5) temperate mountain stream species (Harrison, 1978).

From a taxonomic perspective a number of interesting points can be deduced from a literature survey on the freshwater crustaceans. Certain groups require taxonomic revision, while on the whole, the systematics of a large number of taxa remains confusing, and poorly documented. The systematics of many groups are based on outdated reference material, where only a single specimen (holotype) has been collected from the type locality. However, the majority of systematic studies on these groups have noted that the occurrence of geographic varieties within species makes it difficult to accurately delineate species, and thus discern patterns of endemicity and diversity. The use of modern molecular methods may be particularly helpful in this regard as it has been applied with much success to taxa that are morphologically enigmatic. Noteworthy here, are the allozyme studies by Stewart (1992a, b, c, d) on the amphipod fauna of the south western Cape, South Africa. The latter investigation revealed the presence of 12 new species and two new genera from the area. This study reiterates the fact that the systematic knowledge on the freshwater crustaceans is severely outdated, and further more points to the power of molecular methods to discern species boundaries in aquatic invertebrate groups. The taxonomy of South

African freshwater decapods in particular (crabs, shrimps and prawns) is outdated. As a result a systematic study of freshwater crabs was recently initiated, and forms the focus of the present study.

1.2. General biology and taxonomic history of freshwater crabs of the genus *Potamonautes* (Potamonautidae)

Freshwater crabs, also called river crabs, occur over a wide range of freshwater (rivers, streams, lakes and marshes) and terrestrial (riverbanks, tropical forest floors and deserts) habitats. They are found in tropical, warm temperate to temperate zones of most continental masses (excluding North America and Antarctica) where they are ecologically and economically important, as detritivores and prey items for carnivores and humans (Purves *et al.,* 1991; Hill and O'Keeffe, 1992; Cumberlidge, 1999a). These organisms also act as vectors for deadly diseases such as Paragonimiasis, which affects about 20 million people worldwide, and are important indicators of environmental health and water quality. Freshwater crabs are distinct from their marine relatives as (a) they do not experience a marine milieu at any stage during their life cycle and (b) they lack a planktonic larval phase (they are direct developers).

Ortmann (1897) divided the family Potamonidae into four subfamilies, (1) the Potamoninae Ortmann (1896) (including *Parathelphusa*, H. Milne Edwards, 1853, *Erimetopus*, Rathbun, 1894 and *Potamon*, Savigny, 1816); (2) Deckeniidae Ortmann, (1897) for *Deckenia*, Hilgendorf, 1869; (3) Potamocarcininae Ortmann (1897) (including *Epilobocera* Stimpson, 1860 and *Hypolobocera* Ortmann, 1897, and *Potamocarcinus* H. Milne Edwards, 1853); and (4) Trichodactylinae H. Milne

Edwards, 1853 (including *Trichodactylus* Latreille, 1828, *Orthostoma* Ortmann, 1897, and *Dilocarcinus* H.Milne Edwards, 1853). Ortmann (1897) included all the African freshwater crab species in the Potamoninae and the Deckeniidae. Rathbun (1904, 1905, 1906) included all the freshwater crabs in a single family (Potamonidae) and recognized five subfamilies, of which three (Potamoninae, Gecarcinucinae and Deckeniinae) are found in the Old World. The subfamily Potamoninae included the genus *Potamon* for which Rathbun recognized eight subgenera (based on carapace, cheliped and merus characters), four of which, *Potamonautes*, *Potamon*, *Parathelphusa* and *Geothelphusa,* are African. She recognized 59 *Potamon* species worldwide of which 37 species are African endemics belonging to the subgenus *Potamonautes*. Alcock (1910a, b) accepted Rathbun's broad classification scheme. Bouvier (1917a, 1921) proposed a different classification scheme. He divided the Potamonidae into two groups (Eupotamonea and Parapotamonea) based on the structure of the terminal segment of the mandibular palp, and he recognized four subfamilies of freshwater crab. Colosi (1920) divided the Potamonidae into two groups (Protopotamonida and Eupotamonida) based on the antennule structure. He subdivided the Old World freshwater crabs into three tribes (Potamonini, Propotamonini and Deckenini). Bott's (1955) exceptional monograph on the African freshwater crab fauna represented a significant advance in our knowledge and formed the foundation for many modern works. He recognized three global freshwater crab families, (1) the Potamonidae, (2) Pseudothelphusidae and (3) Deckeniidae. The Potamonidae included four subfamilies, (1) Potamoninae (including *Potamiscus*, *Potamon*, *Potamonautes*, *Sudanonautes* and *Liberonautes*), (2) Gecarcinucinae (for *Gecarcinucus*), (3) Hydrothelphusinae (for *Hydrothelphusa*) and (4) Trichodactylinae

(for *Trichodactylus*) (Bott, 1955). Bott (1955) recognized that four of the five genera in the Potamoninae (*Potamon*, *Potamonautes*, *Sudanonautes* and *Liberonautes)* were uniquely African. He recognized 38 species and 15 subgenera for the *Potamonautes*. Bott (1955) included all the African species into four exclusively African genera. Balss (1957) rejected Bott's (1955) taxonomic conclusions, and assigned all river crabs to a single family (the Potamonidae). Balss (1957) included the African freshwater crabs in the Potamoninae and recognized a number of subgenera of *Potamon* (including *Potamonautes*). Later, Bott (1970a, b) established a new classification scheme for all the families, and recognized three new superfamilies and eleven families.

Bott (1970a) established the Potamonautidae in the Potamoidea and distinguished the Potamonautidae from the Deckeniidae. The Potamoidea is characterized by three characters, a mandibular palp with a simple, undivided terminal segment, afront lacking median frontal teeth and a gonopod 1 with three segments and a long termianl article. The Potamonautidae can be distinguished from the latter family by a two-segmented mandibular palp and a sharply defined postfrontal crest. Monod (1977) did not accept Bott's (1970a, b) classification scheme and recognized two African families of freshwater crabs, the Gecarcinucidae and the Potamidae (the latter included the Potamonautidae). Guinot (1978) recognized a single superfamily, Potamidea that comprises several families. Cumberlidge (1991) included the family Potamonautidae (type genus *Potamonautes*) under the Potamonidea as originally instituted by Ortmann (1897, 1902). The family Potamonautidae (type genus *Potamonautes*) has subsequently been retained by a number of co-workers (Cumberlidge, 1997; Stewart,

1997a, b; 1998). However, the status of the family Potamonautidae has been drawn into doubt as its distinction from the Potamidae remains unclear (Guinot *et al.*, 1997). Although Bott (1970a) recognized eleven families, the number of freshwater crab families worldwide, vary between twelve to eight, depending on the taxonomic authority (Ng, 1988; Ng and Sket, 1996; Rodriguez, 1992; Cumberlidge, 1999a; Dai, 1999). Clearly, the higher classification of freshwater crabs (particularly at the familial level) remain problematic and the topic of much debate.

Currently, a total of four freshwater crab families and a single subfamily are recognized on the African continent (Cumberlidge, 1999a):

- The family **Potamidae** Ortmann 1896 comprise 15 genera and about 60 species, and is distributed in tropical and warm temperate parts of Eurasia to the Philippines and Japan. In Africa this family is confined to the northern tip of the continent (Morroco, Tunisia and Algeria) where a single species occurs. More recently, two new genera from the Island of Socotra off East Africa have been recognised. These are *Socotra* Cumberlidge and Wranik 2002 and *Socotrapotamon* Apel and Brandis 2000.

- The subfamily **Globonautinae** Bott 1969 is endemic to the African continent and comprise two genera (*Afrithelphusa* and *Globonautes*) restricted to the forests of West Africa where they contain four and two species respectively (Cumberlidge, 1999a).
- The family **Deckeniidae** Ortmann 1896 is endemic to Africa and consists of a

single genus *Deckenia,* that comprises two species. This family is known to occur along the East African escarpment (Kenya, Tanzania, Malawi, Somalia) and the island of Zanzibar. The single species of *Deckenia* from the Seychelles, has been re-assigned to a new genus *Seychellum* and transferred to the family Gecaocinucidae (Ng *et al*.., 1995a).

- The family **Platythelphusidae** Colosi (1920) (recently re-established by Von Sternberg and Cumberlidge, 1999a) contains one genus *Platythelphusa* A.Milne-Edwards, 1887, and comprise six species endemic to Lake Tanganyika, East Africa.

- The family **Potamonautidae** is the most specious of the African River crab families. Members of this family are widely distributed throughout the tropical and temperate region of the continent, and certain continental islands (Cumberlidge, 1999a). This family comprises five genera, *Potamonautes* MacLeay, 1838, *Sudanonautes* Bott, 1955, *Liberonautes* Bott, 1955, *Potamonemus* Cumberlidge and Clarke, 1992, and *Louisea* Cumberlidge 1994. However, Cumberlidge (1999a) recently removed the genus *Louisea* from the Potamonautidae and the placement of the genus within any family remains uncertain. Of the remaining genera, the *Potamonemus*, *Sudanonuates* and *Liberonautes* consist of seven, ten, and eight species respectively. They are largely restricted to regions in West and Central Africa (Cumberlidge, 1999a). *Potamonautes* is the most species rich and contains in excess of 60 species and is distributed throughout most of the continent from the Nile delta in Cairo-Egypt to

river systems in South Africa, excluding only the Sahara desert and the Mediterranean fringes of North Africa (Morocco, Algeria and Tunisia). The freshwater crabs of Madagascar also belongs to the Potamonautidae but are all included in a unique subfamily Hydrothelphusinae and currently contain three genera, *Hydrothelphusa* A. Milne-Edwards, 1872, *Madagapotamon* Bott, 1965, and *Skelosophusa* Ng and Takeda, 1994. In addition a forth genus, *Gecarcinautes,* Bott, 1965, belonging to the Gecarcinucinae is also recognised. Cumberlidge *et al.*, (2002) recently described a new genus *Marojejy* from Madagascar. It is noteworthy that this siland has been poorly sampled and probably contains a large diveristry of undesribed taxa.

1.3 A taxonomic overview of the southern African *Potamonautes*

Keppel Barnard, former director of the South African Museum, Cape Town, pioneered the systematics of a large number of invertebrates, and provided the first record on the distribution of freshwater crabs of South Africa, based on previous reports and the results of the Vernay-Lang Expedition (Barnard, 1935). Barnard (1935) noted that all South African species belonged to *Potamon* (subgenus *Potamonautes*). His classification was followed by Chace (1942) and later by Dandy and Ewer (1961). Later, Barnard (1950) reported that at least five freshwater crab species occur in streams, rivers and lakes in South Africa. He remarked that the freshwater crab species of the country were difficult to delineate into well-defined species, as certain populations appeared to exhibit considerable morphological variation. His suspicion that "transitional forms" occur between certain species further complicated the systematics of this group. The high degree of morphological

variation is not unique to the South African freshwater crab fauna, but has also been reported amongst West African freshwater crabs (see Cumberlidge, 1999a).

Thelphusa perlata H. Milne Edwards, 1837, the type species for *Potamonautes* is incidentally also the first South African species to be described. This species is documented to range from river systems and streams in the Western Cape along the south coast as far as Natal, and had been recorded as far as the Democratic Republic of the Congo (although the latter locality is likely to be incorrect due to the occurance of cryptic taxa) (Barnard, 1935, 1950). Krauss (1843) described *P. depressus* (initially *T. depressa*) from specimens collected near Pietermaritzburg as a distinct species. H. Milne Edwards (1853) described a new species, *P. inflatus* from Durban but it was later synonymised with *P. depressus* (Bott, 1955). Rathbun, 1904, described *P. sidneyi* (initially thought to be a variety of *P. perlatus*). *P. sidneyi* is common in river systems and low-lying midlands throughout the Free State (formerly the Orange Free State), Gauteng (formerly Transvaal), KwaZulu-Natal (formerly Natal), the Northern Province and Mpumalanga (Barnard, 1950). The latter species, *P. sidneyi* is morphologically very similar to *P. perlatus.* In 1918, Calman, described *P. warreni* from Potchefstroom, Transvaal (currently North West Province) based on the presence of a series of teeth on the anterolateral margins of the carapace. Barnard (1935) noted that the carapace dentition pattern in this species was characterized by considerable variation, leading him to suspect that it is probably due to hybridization with other freshwater crab species. *P. calcaratus* Gordon, 1929, while it aslo occurred in areas of Mozambique, southern Zimbabwe, and the Kruger National Park (Northern Province). Bott (1955), however, considered *P. calcaratus* to be a subspecies of *P.*

obesus (A. Milne Edwards, 1868). Bott (1960) recorded and described one additional species of freshwater crab, *Gecarcinautes brincki,* from high mountain streams in the Western Cape. He placed this species in a new genus based on what he viewed as the split terminal segment in the mandibular palp of this species. Apart from the cataloguing work on the freshwater crab fauna by Barnard (1950) and more recently by Kensley (1981); the taxonomy of the South African freshwater crabs has not received significant attention recently. The identification keys and subgeneric division proposed by Bott (1955) are now regarded as outdated and *Potamonautes* is in need of revision (Cumberlidge, 1999a).

A freshwater crab-sampling program was initiated in 1992 to investigate the systematics of these macro-invertebrates. This program involved the use of allozyme electrophoresis (a method that has become common practice in systematics) and morphological analyses. During the course of this study, large numbers of preserved freshwater crabs, mainly from museums were examined, dissected and measured. A large number of previously sampled and additional localities were visited and fresh material collected for allozyme electrophoresis. This research has thus far led to the discovery of seven new freshwater crab species from South Africa (table 1.1). The continual discovery of new species from South Africa, clearly demonstrates that this genus of freshwater crabs constitutes a species rich assemblage (e.g. Stewart *et al.*, 1995; Stewart 1997a, b; Daniels *et al.*, 1998; Gouws *et al.*, 2000, 2001).

Among the first new species to be described by Stewart *et al.,* (1995) was *Potamonautes dentatus* from river systems in KwaZulu-Natal. This species is

Table 1. 1. Summary of South African potamonautid crabs described thus far.

Species described	Current name	Taxonomic Authority
Thelphusa perlata	*Potamonautes perlatus*	H. Milne-Edwards (1837)
T. depressa	*P. depressus*	Krause (1843)
P. sidenyi	-	Rathbun (1904)
P. warreni	-	Calman (1918)
P. calcaratus	*P. calcaratus*	Gordon (1929)
P. dentatus	-	Stewart *et al.*, (1995)
Gecarcinautes brincki		Bott (1960)
P. brincki		Stewart (1997a)
P. parvispina	-	Stewart (1997b)
P. unispinus	-	Stewart and Cook (1998)
P. granularis	-	Daniels *et al.*, (1998)
P. clarus	-	Gouws *et al.*, (2000)
P. lividus	-	Gouws *et al.*, (2001)

characterized by strongly denticulate anterolateral margins and the presence of a notch in the postfrontal crest posterior to the exorbital teeth. Later, both Cumberlidge (1996) and Stewart (1997a) independently re-examined the taxonomic status of *Gecarcinautes brincki* Bott (1960) and transferred it to *Potamonautes* based on the similarities of the mandibular palp, the well-developed postfrontal crest, and the threadlike terminal segment of gonopod 2. During 1997, Stewart (1997b) described an additional species, *P. parvispina*, from the Olifants and Berg River systems, Western Cape. This species is endemic to these two river systems and is distinct from any other species in the area by possessing a small epibranchial tooth. In a preliminary study on the Olifants River, it was noted that populations of *P. perlatus* were genetically discreet, but morphologically indistinguishable. This matter was further investigated by Daniels *et al.,* (1999) who reported that a narrow hybrid zone was present between two genetically distinct populations. These authors described a new cryptic species of freshwater crab, *P. granularis*, from the lower reaches of the Olifants River system. Stewart and Cook (1998) described *P. unispinus,* from Mpumalanga. This species is characterized by a single large epibranchial tooth, and a granulated carapace margin.

More recently Gouws (1999) conducted research on the genetic and morphological variation amongst freshwater crabs in KwaZulu-Natal. He showed that *P. depressus* is comprised of two genetically distinct forms and described a new species, *P. clarus* from upper tributaries in the Drakensberg, KwaZulu-Natal (Gouws *et al.,* 2000). Gouws *et al.,* (2001) subsequently described a new species, *P. lividus* from the swamp forests of Kwa-Zulu Natal.

1. 4. Objectives

Whilst these recent systematic endeavors have contributed much to our knowledge of the freshwater crabs of South Africa, many questions remain unanswered. In this section, the key questions are posed along with background information and these key questions will form the basis of this dissertation.

Question 1: What is the relationship among populations in the *P. brincki* species complex?

Stewart (1997a) showed that *P. brincki* is a highly structured genetic entity. She reported a genetic identity (*I*) of 0.67 between the two main population groups on the Cape Peninsula and the Hottentots Holland Mountains. These two population groups are also morphologically distinct. Specimens from the Cape Peninsula did not have a flange on the terminal segment of the mandibular palp and the terminal segment of the first pleopod was different in shape to those collected on the Hottentots Holland Mountains. Stewart (1997a) recommended further sampling between the two main population groups in order to assess the population structure and systematic position of the Table Mountain populations. The results of this study are presented in chapter 3. This study was published as Daniels *et al.*, (2001a).

Questions 2: What are the morphological features that distinguish *P. warreni* from other freshwater crabs in Southern Africa? Is *P. unispinus* a distinct species or simply a morphological variety of *P. warreni* or are these two species hybridizing extensively? Does the variation in the dentition patterns of

P. warreni follow a distinct pattern and how reliable are tooth patterns in distinguishing freshwater crab species?

Calman's (1918) original description of no more than two pages for *P. warreni* made no reference to the taxonomically important gonopods or chelipeds. Consequently the species warrants redescription. Barnard (1935) noted that *P. warreni* varies in carapace dentition from a typically form characterised by 5-10 teeth to a form with a single small blunt tooth on the anterolateral margin of the carapace. He reported that certain *P. warreni* specimens from populations in southern Africa (Barberton, Lake Chrissie, Salisbury- Harare and Chishawasha) are characterised by fine and regularly decreasing serrations of the carapace margins. Barnard (1935) further noted that some of the feebly dentate forms of *P. warreni* appear very similar to either *P. perlatus* (H. Milne Edwards, 1837) or *P. sidneyi* (Rathbun, 1904) and were particularly difficult to separate. This led Barnard (1935) to suspect that these taxa may be hybridising. The feebly dentate form described by Barnard (1935) as a variety of *P. warreni*, was, however, recently described as a new species, *P. unispinus,* by Stewart and Cook (1998). Preliminary allozyme data (Stewart and Ridgeway. pers comm.) for *P. warreni* revealed limited genetic differences between this species and *P. unispinus*. This sparked a debate as to the systematic status of *P. unispinus*. Results of this study are presented in chapters 4 and 5. These studies have been published as Daniels *et al.*, (2001b) and Daniels (2001c).

Question 3: What is the status of intermediate hybrid populations?

Gouws (1999) reported extensive genetic differentiation existing between *P. depressus* and *P. clarus*. He noted that a number of hybrid populations occur between these two species. The hybrid zone exhibited variation in both genetic and morphological characters and certain populations maintain a number of unique alleles that suggested that the taxa from intermediate localities may be the product of hybridization that arose after secondary contact between the two species. Gouws (1999) further noted that the presence of abrupt changes in allele frequencies between populations provides evidence for a probable ancient zone of hybridization and introgression between these two taxa. He pointed out that populations over the entire distribution of the two species should be sampled and the patterns of genetic variation assessed using mitochondrial (mt) DNA markers. The results of this study are presented in chapter 6.

Question 4: Are populations of *P. calcaratus* genetically more structured, relative to other freshwater crab species studied to date?

P. calcaratus was first described by Gordon in 1929. *P. calcaratus* is unique among the south African freshwater crab species as it is restricted to waterholes and appears to have very specific habit requirements, and thus occur in isolated habitats. The discontinuity in the distribution of this species is thought to result in populations that are genetically structured, however this hypothesis remains untested. Results from this study are presented in chapter 7.

1. 4. The phylogenetic origin of the southern African freshwater crabs

Questions 5: Are freshwater crabs useful zoogeographic indicators? What are the phylogenetic relationships, and the importance of hybridization amongst the southern African freshwater crab species? How valid are Bott's subgeneric divisions?

The phylogenetic relationships among the South African potamonautid crabs has received no attention, apart from Bott's (1955) hypothesis. His study was based on a limited number of morphological characters derived from carapace, gonopod and mandibular palp characters that are now known to exhibit considerable variation. Consequently, his phylogeny may reflect an inaccurate genealogy of the group, as the morphological characters may not be homologous structures and may thus not contain phylogenetic signals. Bott's study recognized three distinct subgeneric divisions for the South African species described until 1955. According to this study, group one was comprised of *Potamonautes* belonging to the subgenus *Potamonautes* and included *P. warreni, P. bayonianus, P. dubius* and *P. perlatus.* Group two included species of the subgenus *Obesopotamonautes* (*P. langi*, *P. obesus* and *P. calcaratus*) and group three included species of the subgenus *Orthopotamonaute* (*P. depressus, P. dybowskii* and *P. sidneyi*). The relationship of the newly described river crab species remain unclear within the hypotheses proposed by Bott (1955), and in relation to other African freshwater crabs in *Potamonautes*.

Kensley (1981) suspected that the occurrence of diffuse species boundaries might be

evidence to support Bott's (1955) subgeneric divisions. Kensely (1981) reported that where river systems are "defined" (isolated from other large river systems) species boundaries among freshwater crabs are well defined. However, where river systems are poorly defined and characterized by river capture or geological changes the freshwater crab fauna usually comprise closely related species that may hybridize. The freshwater crabs of South Africa exhibit very interesting distribution patterns, as certain species are confined to high mountain streams - these are usually endemic - while other species are distributed over a wide geographic range and are common in the low-lying river systems, and yet others are adapted to a terrestrial mode of life. The construction of a phylogeny for this group will provide an understanding of the factors that have determined historical patterns of diversification and aid in our understanding of the biogeography of the South African freshwater crab fauna. More recently a dispute has arisen in the literature on the utility of freshwater crabs as zoogeographic indicators. Banarescu (1990) is of the opinion that freshwater crabs are poor biogeographic indicators. However, Ng and Rogdriguez (1995) argue that Banarescu's conclusions are ill founded and not a true reflection of the importance of freshwater crabs as zoogeographic indicators. Results from this study are presented in chapter 8.

Chapter 2

2. 1. A review of the analytical methods used in this study

Systematic studies have for centuries interested biologists. Traditionally systematic endeavors were based on morphological features. The use of morphological data in systematics is now common place and has been used to document and describe the history and diversity of living and extinct organisms. Fixed morphological characters between taxa are frequently used as diagnostic characters for delineating species boundaries. Morphological data sets may, however often be prone to environmental plasticity and diagnostic characters may be highly variable within taxa. In such instances the assumed fixed morphological characters have limited value in distinguishing taxa. In addition, morphologically indistinguishable (cryptic) forms, assumed to be a single species, may often be comprised of genetically differentiated (sibling) species (Knowlton, 1993). Where systematic controversies are evident, a combination of morphological and genetic studies is often useful to determine the systematic affinities of the taxa under scrutiny (Moritz and Hillis, 1996). The use of morphological studies have been revitalized in recent years (Rohlf and Marcus, 1993) and these arguably remain a vital source of information in systematics and cladistics (Hillis, 1987). Phylogenetic studies derived from morphological characters have provided many of the first insights into understanding relationships among organisms. However, as these characters may often be few and subjected to evolutionary convergence, they may not provide a true reflection of the relationship among species. With the advent of molecular techniques, phylogenies derived from morphological characters could often be re-examined and either corroborated or rejected.

Molecular markers such as proteins (total protein, serological studies and allozymes) (e.g. Hedgecock *et al.,* 1982; Busack, 1988, McMillen-Jackson *et al.,* 1994; Merkouris *et al.,* 1998); DNA sequencing (e.g. Bucklin *et al.,* 1996 a, b, 1997; Schubart *et al*, 1998, 2000); restriction fragment length polymorphism (RFLPs) (Grandjean *et al.,* 1998); rapid amplification of polymorphic DNA (RAPD' s) (D'Amato and Corach, 1996, 1997); amplified fragment length polymorphism (AFLP' s), and more recently microsatellites or simple sequence repeat (SSR) have become common practice in the study of evolutionary biology. These methods have become invaluable in answering questions pertaining to population genetic structure, general systematics and phylogenetics.

Over the past 30 years allozyme electrophoresis has become a popular tool in the study of evolutionary biology since its discovery in the early 1960's. Classic undertakings by Harris (1966) and Hubby and Lewontin (1966) paved the way for a revolution in biochemical systematics. This method identifies similarities and differences in allozyme structure and can be used to identify species and test biogeographic hypotheses. The variation encoded in allozymes provides a relative measure of the variation encoded in DNA (Grant *et al.*, 1989). In decapod crustaceans, allozymes have generally been shown to exhibit low levels of variability within species (Hedgecock *et al.*, 1982; Autsuka *et al.,* 1995; Creasy *et al.,* 1997; Merkouris *et al.,* 1998; Daniels *et al.,* 1998, 1999). Allozymes are conservative markers and most studies sample a relatively small proportion of the total genome, and thus can fail to detect genetic structure. The chief disadvantage is that material has to be stored at below zero temperatures for electrophoresis, to prevent protein

denaturation. In addition the scoring of the gels is often subjective and electropheretic bands that appear at the same level on the gel may not be homologous. Despite recent advances in molecular genetics, allozymes remain a practical tool that is, less costly, fast, and a relatively easy means to study evolutionary questions (e.g. Avise, 1994; Hillis and Moritz, 1996). Allozyme electrophoresis is less useful for phylogenetic studies due to the questionable homology among presumptive loci (Swofford *et al.,* 1996; Wiens, 2000a).

The late 1980's to early 1990's have seen a dramatic shift in molecular approaches. Among the methods that were applied with great success are RFLPs (Zwanenburg *et al.,* 1992). This method allowed for the digestion of mt DNA with endonucleases. RFLP's have been used to quantify the degree of divergence in population genetic studies of a wide range of organisms, including crustaceans (Grandjean *et al.,* 1998). The study of genetic variation at the nucleotide level, through direct sequencing of DNA (mitochondrial or nuclear genes) has yielded the "ultimate" level of resolution. A number of technical advances have led to the widespread use of DNA sequencing in evolutionary studies. The polymerase chain reaction (PCR) (Saiki *et al.,* 1985, Mullis *et al.,* 1986) allowed for direct sequencing of amplified gene portions (from miniscule amounts of source DNA) without an intermediate cloning step. The automation of sequence protocols and the use of "universal" primer pairs, that obviate the need for prior sequence data (Kocher *et al.,* 1987; Innis *et al.,* 1990). Sequence data have become popular because an unlimited number of sequence characters are potentially available, and these data are applicable to both closely and distantly related taxa. The rapid accumulation of DNA sequence data over the past decade has led to the

discovery that some genes are inherently better at answering particular questions pertaining to population genetics, systematics and phylogenetics. This can be attributed to the fact that genes and sites within genes evolve at different rates because of variation in the level of functional constraints. The choice of markers should thus not be arbitrary but dependent on the research endeavor at hand.

Mitochondrial genes were among the first genes that were used with resounding success, probably because of the high number of copies per cell making DNA isolation easy. In addition, the absence of recombination makes it a powerful tool for use in evolutionary biology (Brown, 1985). Among the genes most frequently used are protein coding and ribosomal genes. The sequencing of protein coding genes (e.g. cytochrome-oxidase sub-unit 1 (COI), cytochrome b (Cyt b), NADH dehydrogenase subunit 6 (ND 6) are good for divulging information on population structure and to discern relationships at the family and species level (Simon *et al.*, 1994). On the other hand, ribosomal genes (12 S and 16 S rRNA mtDNA) are useful to study phylogenetic relationships among and within a wide spectrum of taxa. Among the mitochondrial genes, the protein coding genes (e.g. COI, COII and Cyt b) are relatively easy to align, exhibit few complex mutations, have a predictable pattern of substitution and a high rate of silent (3rd codon position) sites. The protein-coding gene COI is a rapidly evolving mitochondrial gene and has been used to study evolutionary relationships among closely related species (Bucklin *et al.,* 1996 a, b, 1997).

The 12 S rRNA gene is known to evolve at a moderate rate and has been extensively

used to study phylogenies at the sub-species, species and genus level. Both the 12 S rRNA and 16 S rRNA subunits have been successfully used to study phylogenetic relationships, in a variety of organisms including crustaceans (Cunningham *et al.,* 1992; Bucklin *et al.*, 1996a, b, 1997; Schubart *et al.,* 1998, 2000). Sequence data are also characterized by a multitude of problems, for example ancestral polymorphisms in genes, the occurrence of pseudogenes, and rate heterogeneity within genes, to name but a few. It would appear that phylogenies that are derived from a multitude of independent character sets (morphology, allozymes and sequencing) provide the best estimates of evolutionary relationships.

Chapter 3

Geographic patterns of genetic and morphological divergence amongst populations of a freshwater crab (Decapoda, Potamonautidae) with the description of a new species from mountain streams in the Western Cape, South Africa

Abstract

Recent systematic research revealed that *Potamonautes brincki* is comprised of two genetically and morphologically distinct population groups. The systematic affinities between these population groupings have remained uncertain. In the present study the relationship between the population groups was examined. Eleven populations were collected from high mountain streams in the Western Cape, South Africa and used in genetic and morphological analyses. Allozyme electrophoresis of 13 protein coding loci separated two main population groups, group A (Cape Peninsula groups) and B (Hottentots Holland) at $I = 0.73$. Two additional genetic groupings were evident with group B being conspecific to group A, and group D being conspecific to group C. Morphological examination of pleopod 1 and the terminal segment of the mandibular palp showed considerable differences between the two main population groups, with group A and B being similar and group C and D being similar. The morphometric data for the four main groups were examined using a discriminant functions analyses and the two main groups compared using analyses of covariance. Discriminant functions analyses showed a moderate degree of overlap between the groups. Additional morphometric data showed a clear discrimination between the two main population groups. The genetic and morphometric data sets exhibited congruent patterns of variation and the data show the presence of a species boundary. A new

freshwater crab species, *P. parvicorpus* sp. n. is described. The results of the present study are discussed in the light of historic and contemporary factors that were likely to have contributed to speciation.

Introduction

Four river crab species are known to occur in the Western Cape, South Africa. *Potamonautes perlatus* (H. Milne-Edwards, 1837) has until recently been thought to be the only river crab species that occurs in this area, where it is widespread and occurs from Clanwilliam in the Western Cape to Port Elizabeth in the Eastern Cape, and typically occurs in upper, middle and lower reaches of rivers (Barnard, 1950). The remaining three species are endemic to the region. *Potamonautes granularis* (Daniels *et al.*, 1998b) is known only from the lower reaches of the Olifants River system. *Potamonautes parvispina* (Stewart, 1997a) is endemic to high mountain streams in the Berg and Olifants River systems, while *P. brincki* (Stewart, 1997b) occurs in high mountain streams on the Cape Peninsula, the Hottentot's Holland Mountains and the Kleinriver mountains. These results indicate that the freshwater crab fauna of the region is considerably more species rich than previously thought (Barnard, 1950). With the exception of *P. brincki*, studies of the population genetics of these three crab species have revealed the general absence of population structuring (Daniels *et al.*, 1998a, 1999a).

Stewart (1997b) reported substantial genetic and morphological divergence amongst allopatric populations of *P. brincki* and identified two distinct population groups. One group occurred on the Cape Peninsula, mainly on Table Mountain with a second group occurring further eastwards on the Hottentots Holland Mountains and the Kleinriver Mountains. Allozyme data demonstrated that *P. brincki* is a highly structured genetic entity (genetic identity, $I = 0.75$) with substantial fixed allelic

differences between these two population groups. The extent of the genetic differentiation recorded amongst *P. brincki* populations is exceptional. Genetic differentiation within freshwater crab species has been found to be low, usually in the order of 0.88 to 1.00 (Daniels *et al.,* 1999b). The results obtained by Stewart (1997b) thus possibly provide evidence for a species boundary between these *P. brincki* populations.

Morphologically these two groups exhibit variation in the structure of the mandibular palp and the terminal segment of pleopod 1 (gonopod). The Cape Peninsula populations all have a simple terminal segment of the mandibular palp, which is covered by a dense tuft of setae (Stewart, 1997b). However, in specimens from the Hottentots Holland and the Kleinriver Mountains, the terminal segment of the mandibular palp is divided and characterized by a dense tuft of setae that arises from a ridge or a flange. The inner margin of the subterminal segment of pleopod 1 is markedly irregular in the Cape Peninsula populations and appears to differ in shape from those of the Hottentots Holland and Kleinriver Mountains. These differences, along with the strong genetic discontinuity led Stewart (1997b) to suggest that a species boundary may be present between these two population groups. However, she suggested that before such a species boundary could be confidently identified, further samples need to be collected from the region that is geographically situated between these two main population groups.

The primary objectives of the present study are fourfold. Firstly, to investigate the genetic relationship of populations geographically intermediate to the two main

groups defined by Stewart (1997b). Secondly, to examine the structure of the terminal segment of the mandibular palp and the structure of pleopod 1 in all populations. Thirdly, to examine population structuring within and between population groupings and fourthly to revisit the possibility of the existence of a species boundary between the Cape Peninsula and the Hottentots Holland groups.

Materials and methods

Sample collection

Specimens were collected from 11 first and second order mountain streams in the Western Cape (fig. 3. 1). The geographically intermediate populations were collected from the Lang River, a tributary of the Eerste River (Jonkershoek), and unnamed tributaries of the Lourens and the Sir Lowry's Rivers. In addition, samples were recollected from the same localities on the Cape Peninsula, Hottentots Holland Mountains and Kleinriver Mountains (Fernkloof) as used by Stewart (1997b). Voucher specimens were deposited at the South African Museum (SAM), Cape Town, South Africa. Crabs were caught with handnets following their attraction to baited (ox heart) lines. On capture, crabs were transferred alive to the laboratory and killed by freezing at -20 or -80 °C for 24 h prior to measurement and tissue extraction.

Genetics

Muscle tissue was removed from each specimen after all morphological measurements had been taken. Tissue samples were placed in cryotubes and stored in liquid nitrogen. The genetic variation at 18 isozyme loci was examined on a 13% arch gel (Sigma Chemicals Co., St Louis, Missouri, USA). Only 13 allozyme loci were

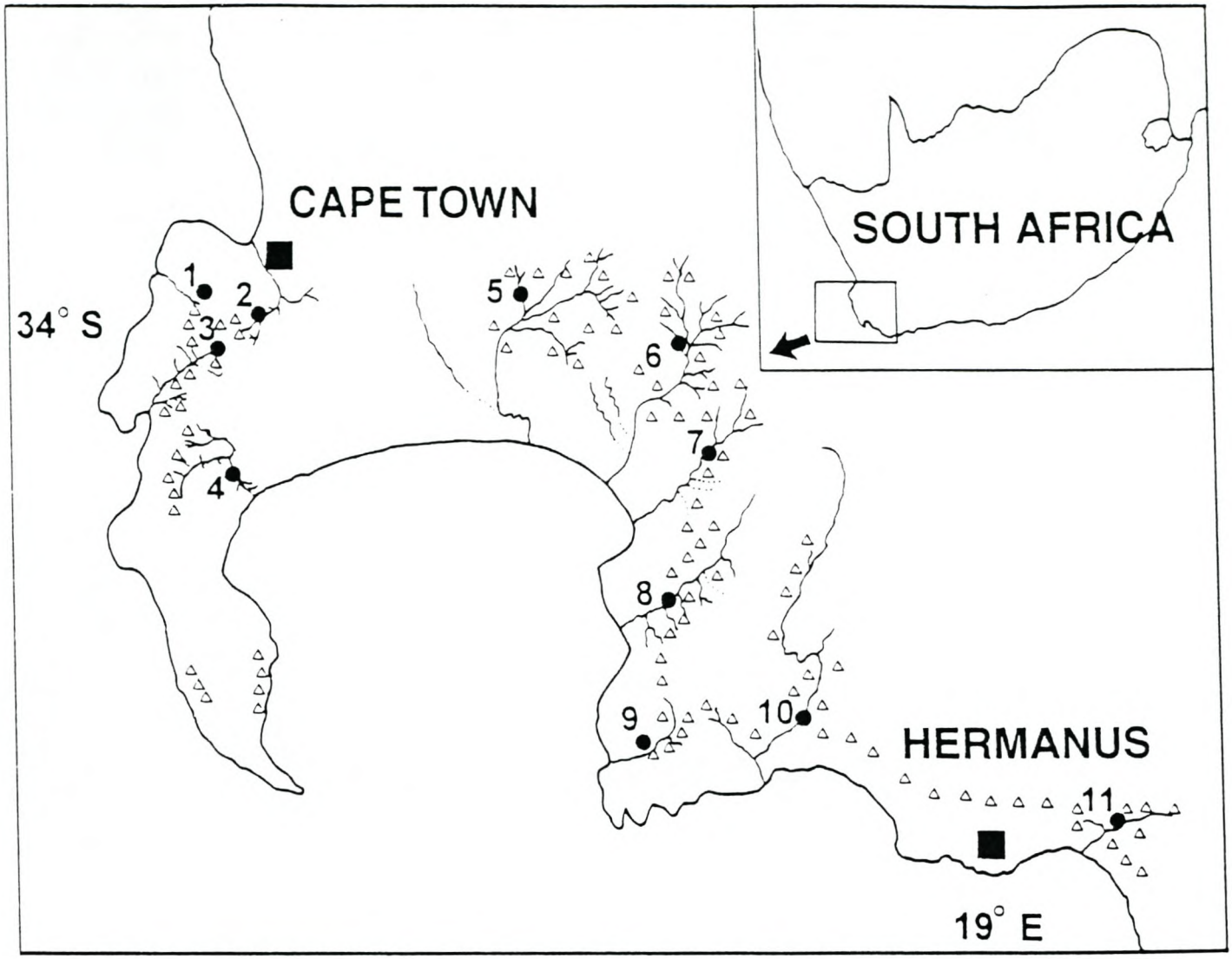


Figure 3. 1. Localities sampled: 1, Blinkwater; 2, Orange kloof; 3; Kirstenbosch; 4, Silvermine; 5, Jonkershoek; 6, Lourens River; 7, Sir Lowry's River; 8, Steenbras; 9, Rooiels; 10, Palmiet and 11, Fernkloof. Open triangles represent mountain ranges. scored for all the populations, and analysis has thus been confined to these.

consistently scored for all the populations, and analysis has thus been confined to these. Three electrophoretic buffer systems were used: (A) a discontinuous tris-citrate-borate-lithium hydroxide buffer, gel buffer pH 8.7; electrode buffer pH 8.0 (Ridgeway *et al.*, 1970), (B) a continuous tris-borate-EDTA buffer system; gel and electrode buffer at pH 8.6 (Markert and Faulhaber, 1965), and (C) a continuous tris-citrate buffer system, gel and electrode buffer at pH 6.9 buffer (Whitt, 1970).

Tissue samples were placed in Eppendorf tubes and homogenised in 0.01 M Tris buffer (pH 8). Water-soluble proteins were separated from the homogenate by centrifugation at 2500 g for 5 min. Filter paper (Whitman's # 3) wicks were dipped into the supernatant and the wicks were inserted into a horizontal starch gel. Red food colour dye was used as a marker on gels. Gels were run for between 2.5 to 5 h at 30-50 mA, at 4 °C in a fridge. Thereafter, gels were divided into 3-4 slices, and stained for enzymatic activity by applying specific chemical reagents in a 2 % agar overlay (Shaw and Prasad, 1970). The enzymes stained for and the buffer system used are provided in table 3. 1. The numerical analyses were performed using the BIOSYS-1 programme (Swofford and Selander 1981). Allelic and genotype frequencies were computed. Chi-square analyses were used to test if populations were in Hardy-Weinberg equilibrium. Levene's (1949) correction for small sample size was used. The mean heterozygosity per locus (Ho) for each population was calculated using Nei's (1978) unbiased estimates. The percentage of polymorphic loci in each population was determined. Loci were considered polymorphic if the frequency of the most common allele did not exceed 0.99.

Table 3. 1. Enzyme and buffer systems used during electrophoresis. N = the number of loci.

Enzyme	Abbreviation	Buffer	E.C.number	N
Arginine kinase	*Ark-1*	A	2.7.3.3	1
Glucose phosphate isomerase	*Gpi-1*	A	5.3.1.9	1
Isocitric dehydrogenase	*Idh-1, 2*	B	1.1.1.42	2
Lactate dehydrogenase	*Ldh-1*	B	1.1.1.27	1
Peptidase (Glycyl leucine as substrate)	*Gl-1*	B	3.4.11-	1
Peptidase (Leucine tyrosine as substrate)	*Lt-1*	A	3.4.11-	1
Malate dehydrogenase	*Mdh-1, 2*	C	1.1.1.37	2
Malic enzyme	*Me-1*	C	1.1.1.40	1
Mannose phosphate isomerase	*Mpi-1*	C	5.3.1.8	1
Phosphoglucomutase	*Pgm-1, 2*	A	2.7.5.1	2

The mean unbiased genetic identity (*I*) among the populations were calculated from the allelic frequencies according to Nei (1978).

Values of $F_{(ST)}$ can be considered to be a measure of the proportion of the genetic variation among populations. Little genetic differentiation is evident when the $F_{(ST)}$ value falls between 0 to 0.05 , moderate genetic differentiation is indicated by values between 0.05 to 0.15, while values between 0.15 to 0.25 indicates great genetic differentiation and values > 0.25 very great genetic differentiation (Wright, 1978). The F statistics, including $F_{(IS)}$ (the mean value of genetic differentiation or inbreeding coefficient within subgroups), $F_{(IT)}$ (the mean value of genetic differentiation over the entire population) and $F_{(ST)}$ (the genetic differentiation between any two subpopulations), were calculated to determine the degree of genetic differentiation amongst the populations (Wright, 1965). $F_{(ST)}$ values were tested for significance using the formula given in Waples (1987), $\chi^2 = 2\,N\,F_{(ST)}\,(k - 1)$, where N = the total number of individuals sampled and k = number of alleles at the locus. The degrees of freedom are equal to $(k - 1)\,(r - 1)$, where r is the number of populations. Levels of differentiation were examined between the Cape Peninsula group and the Hottentot's Holland and the Intermediate groups. The Hottentots Holland group was in turn compared to the Kleinriver Mountains population (Fernkloof) and to the Intermediate group. In addition genetic differentiation within the Cape Peninsula group (Blinkwater, Silvermine, Kirstenbosch and Orange kloof), the Intermediate group (Jonkershoek, Lourens River and Sir Lowry's Pass) and the Hottentots Holland group (Steenbras, Rooiels and Palmiet) were examined. The mean $F_{(ST)}$ for all the polymorphic loci was calculated across all the 11 populations.

Morphometrics

The carapace and limbs were measured to the nearest 0.1 mm using a digital caliper attached to a portable computer. The following carapace measurements were taken: the carapace length measured along the medial line (CL); the carapace width at the widest part (CWW); the width of the posterior margins of the carapace (CWP); distance between the postfrontal crest and the anterior margins of the carapace (PFCD); the distance between the medial margins of the orbits (ED); the distance between the exorbital teeth (CWA) and the carapace height (depth) (CH). The following non-carapace variables were measured; the length of the propodus of pereopod 2 (P2PL); the width of propodus of pereopod 2 (P2PW); the length of merus of pereopod 2 (P2ML) and the width of merus of pereopod 2 (P2MW); the length of the propodus of pereopod 5 (P5PL); the width of propodus of pereopod 5 (P5PW); the length of merus of pereopod 5 (P5ML) and the width of the merus of pereopod 5 (P5MW). The carapace and pereopod variables were analyzed separately using discriminant functions analysis following log transformation of the variables. Groups were defined according to the genetic clusters evident from the genetic analysis. The jack-knife method was followed to calculate the classification functions. Bivariate scatter plots were drawn based on selected carapace and non-carapace variables between the 2 putative species identified in this study. The slopes were statistically compared using analysis of covariance (ANCOVA) in the STATISTICA software package (Stat Soft Inc., 1996).

Qualitative analyses

Pleopod 1 and the structure of the mandibular palp were examined in all populations. Figures were drawn from samples representing each of the four population groups using a camera lucida attached to a Wild stereo microscope.

Results

Genetics

The allele frequencies for the seven polymorphic loci are presented in Appendix 1. Of the 13 loci examined, six were monomorphic (*Idh-2, Mdh-1, Idh-1, Lt-2, Mpi-1* and *Pgm -2*). The seven polymorphic loci were *Ark-1, Gpi-1, Me-1, Mdh-2, Ldh-2, Pgm-1* and *Gl-1*. The number of alleles ranged from two in *Ark-1, Me-1, Mdh-2, Ldh-2, Pgm-1*, to four in *Gl-1* and *Gpi-1*. No single locus was polymorphic in all the populations. Of the 18 polymorphic cases, seven (38 %) were out of Hardy-Weinberg equilibrium ($P < 0.05$), which was attributed to a deficit of heterozygotes containing rare alleles. The following populations were out of Hardy-Weinberg equilibrium for the locus identified: *Gl-1* ($\chi^2 = 83.01$) in Blinkwater; at *Me-1* ($\chi^2 = 7.2$) and *Gl-1* ($\chi^2 = 5.67$) in Orange kloof; at *Gl-1* ($\chi^2 = 9.42$) in Jonkershoek; at *Gl-1* ($\chi^2 = 9.14$) in Lourens River; *Gl-1* ($\chi^2 = 44.31$) in Rooiels; and at *Mdh-2* ($\chi^2 = 12.08$) and *Gl-1* ($\chi^2 = 26.78$) in Fernkloof. The mean number of alleles ranged from 1 to 1.3 and the percentage of polymorphic loci range from 0 to 30.8 %, while the heterozygosity values ranged from 0 to 0.053 (table 3.2).

The allele frequencies were used to determine the degree of genetic similarity between populations. The genetic similarity between the populations was used to construct

Table 3. 2. The mean number of alleles per locus, the percentage of the loci that were polymorphic and the mean observed heterozygosity (Ho) amongst the 11 populations.

Population	Mean sample size	Mean number of alleles per locus	Percentage of loci polymorphic	Ho
1. Blinkwater	43.5	1.2	23.1	0.008
2. Orange kloof	7.8	1.3	30.8	0.050
3. Kirstenbosch	7.2	1.1	7.7	0.038
4. Silvermine	12.7	1.2	15.4	0.053
5. Jonkershoek	25.0	1.2	15.4	0.010
6. Lourens River	20.0	1.1	7.7	0.006
7. Sir Lowry's Pass	22.5	1.1	7.7	0.013
8. Steenbras	5.0	1.0	0.0	0.000
9. Rooiels	18.6	1.2	7.7	0.007
10. Palmiet	9.5	1.0	0.0	0.000
11. Fernkloof	31.4	1.3	23.1	0.020

a UPGMA dendrogram. The dendrogram separated the populations into two distinct clusters, at $I = 0.73$ (fig. 3. 2) coinciding with the geographic distribution of the populations and it was clear that two main groups could be discerned. Three loci, *Ark-1, Gpi-1* and *Me-1* were fixed for alternative alleles in these two population groups. Each of these two major clusters in turn consisted of two distinct subclusters. Genetic identity values within each of these two subclusters were > 0.88. The intermediate group (Group B) separated from the Cape Peninsular group (Group A) at $I = 0.89$, while the Fernkloof population (Group D) separated from the Hottentots Holland (Group C) group at $I = 0.92$.

Population substructuring as derived from the $F_{(ST)}$ values was calculated (table 3. 3). When groups A and C were compared, highly significant $F_{(ST)}$ values were obtained at all loci, ranging from 0.019 to 1.00, with a mean of 0.825. Similarly when group A and group B were compared, highly significant $F_{(ST)}$ values were obtained and ranged from 0.019 to 1.00 with a mean of 0.655. A comparison between groups B and C was significant for all loci, ranging from 0.087 to 1.00 with a mean of 0.899. Group C and group D showed significant $F_{(ST)}$ values, that ranged from 0.033 to 1.00 with a mean of 0.659. Values of $F_{(ST)}$ for the four Cape Peninsula populations (Group A) were considerable, with *Me-1, Ldh-2, Pgm-1* and *Gl-1* showing significant $F_{(ST)}$ values ranging from 0.024 to 0.692. In the geographically intermediate group (group B) there was limited genetic structuring, and $F_{(ST)}$ values ranged from 0.043 to 0.068 with *Gl-1* being statistically significant. In the Hottentot's Holland population group (group C), moderate genetic structuring was evident, and a significant result was obtained for *Gl-1* with a mean $F_{(ST)}$ of 0.085. The pairwise $F_{(ST)}$ amongst all the eleven populations

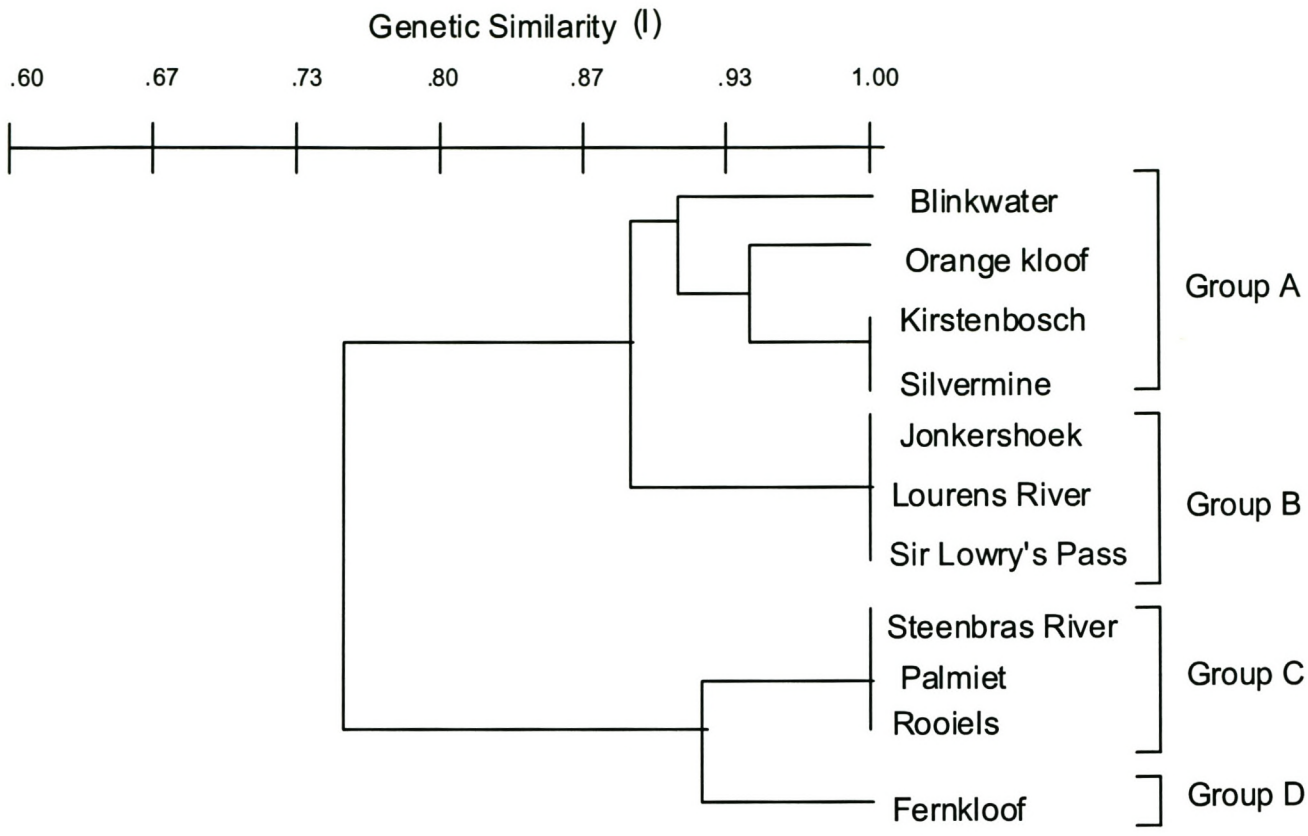


Figure 3. 2. UPGMA analysis based on Nei's genetic distance.

Table 3. 3. $F_{(ST)}$ values within and amongst the three population groupings. (N. S = not significant $P > 0.05$, * $P < 0.05$; **$P < 0.01$ and ***$P < 0.001$).

	Locus							
Group	*Ark-1*	*Gpi-1*	*Me-1*	*Mdh-2*	*Ldh-2*	*Pgm-1*	*Gl-1*	Mean
Cape Peninsula and Hottentots Holland Group	1.00 ***	0.982 ***	0.892 ***	0.019 N.S	0.407 ***	0.688 ***	0.623 ***	0.825 ***
Cape Peninsula and Intermediate group	1.00 ***	0.047 **	0.720 ***	0.019 N.S	0.407 ***	0.159 ***	0.570 ***	0.655 ***
Intermediate group and Hottentots Holland group	-	0.938 ***	1.00 ***	-	-	1.00 ***	0.087 **	0.899 ***
Hottentots Holland group and Fernkloof	1.00 ***	0.033 N.S	-	0.122 ***	-	-	0.065 ***	0.659 ***
Cape Peninsula	-	0.024 N. S	0.692 ***	0.017 N.S	0.375 **	0.080 *	0.668 ***	0.485 ***
Intermediate group	-	0.043 N.S	-	-	-	-	0.068 *	0.061 ***
Hottentots Holland	-	-	-	-	-	-	0.085 ***	0.085 ***
Overall	1.00 ***	0.936 ***	0.931 ***	0.129 ***	0.421 ***	0.798 ***	0.571 ***	0.838 ***

ranged from 0.129 (*Mdh-2*) to 1.00 (*Ark-1*), with a mean at 0.838. This indicates considerable genetic differentiation between the populations. The mean $F_{(IS)}$ value between all the eleven population groups was 0.367. $F_{(IT)}$ ranged from 1.00 (*Ark-1* and *Me-1*) to 0.524 (*Mdh-2*) with a mean of 0.897.

When geographic distance (km) between populations sites was plotted against genetic identity (*I*) values for the two main population groups (Fig. 3. 3), a clear relationship existed between genetic and geographic distance for the Cape Peninsula and the intermediate groups (1) ($Y = 0.0467X + 0.002$; $r = 0.026$; $P < 0.05$). However, no genetic patterns exist with geographic distance for the Hottentot's Holland and Fernkloof group (2) ($Y = 0.372X - 0.024$; $r = -0.026$; $P > 0.05$).

Morphology

The two-dimensional plot of the individual scores along the first two canonical variables based on the log transformed carapace measurements for the four genetically defined population groups showed that four distinct groupings are present (Fig. 3. 4). The first two canonical variables contributed 90.03 % to the total variation between groups (table 3. 4). The classification functions for the four population groups are presented in table 3. 5, and give a moderate degree of support that the four genetically distinct population groups can be distinguished using morphometric criteria. In addition, a two-dimensional plot of the individual scores along the first two canonical variables based on the log transformed pereopod measurements for the four-population groups showed differentiation (fig. 3. 5).

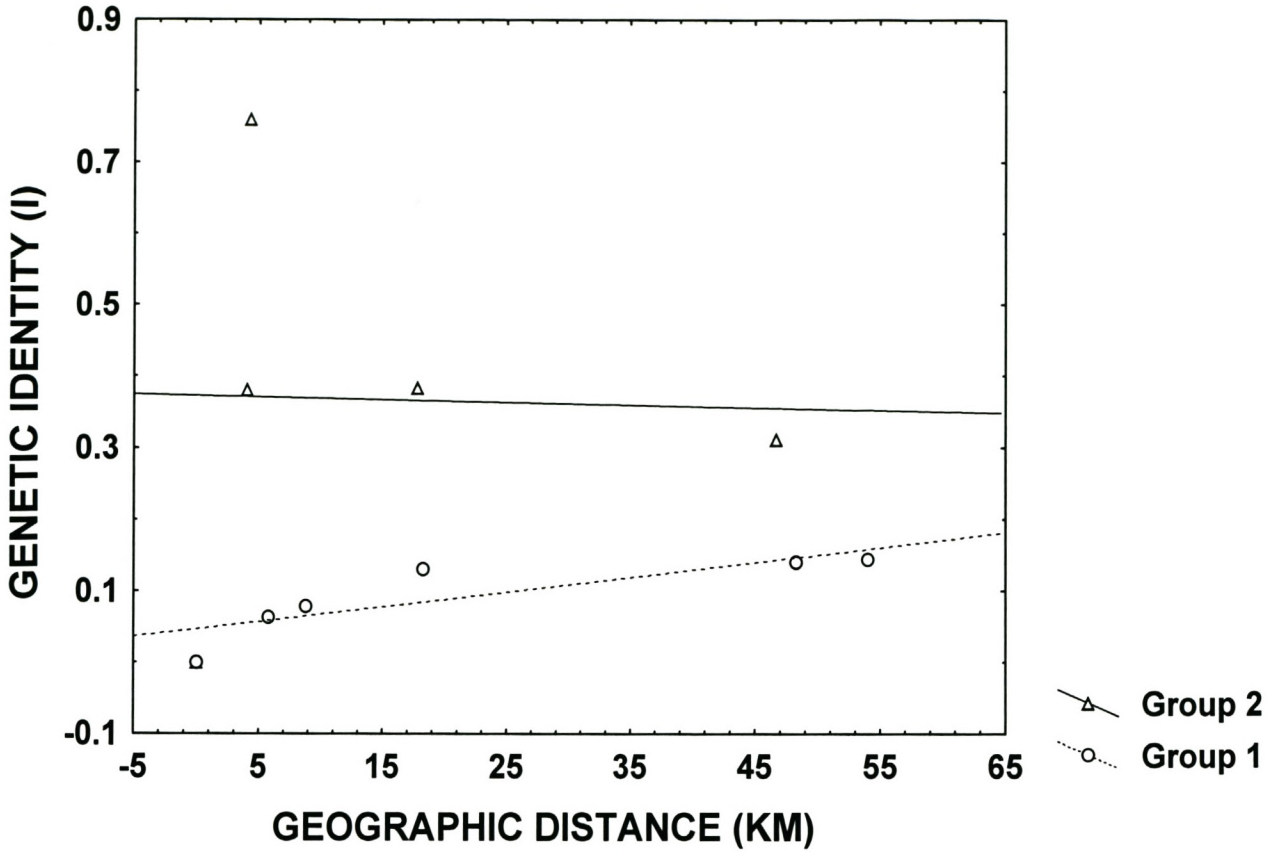


Figure 3. 3. Scatterplot of geographic distance (km) against Nei's (1978) genetic distance.

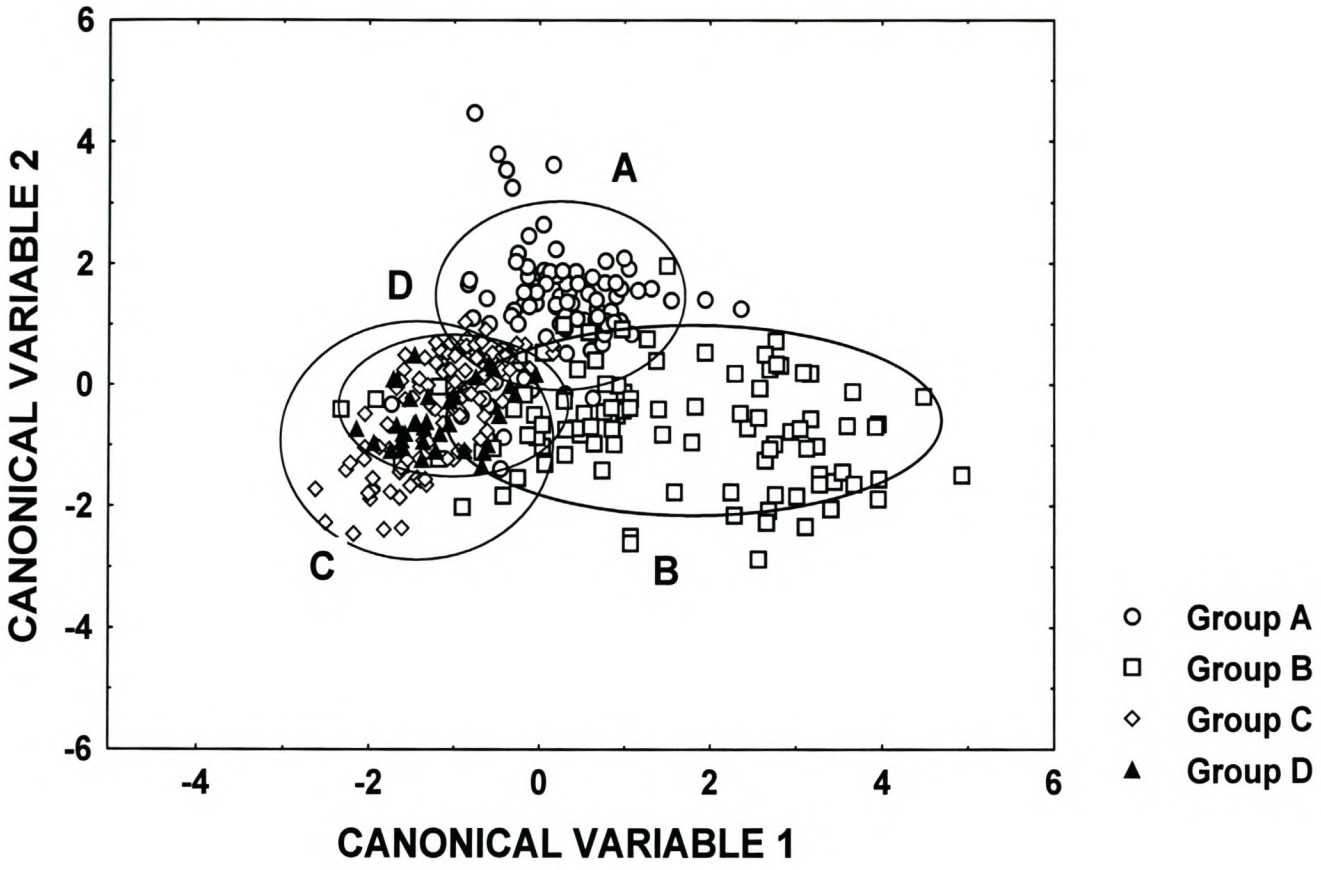


Figure 3. 4. Polygons encompassing the individual scores for the four genetically distinct population groups based on the log transformed carapace variables. Group A (Cape Peninsula group); Group B (Intermediate group); Group C (Hottentots Holland group) and Group D (Fernkloof population).

Table 3. 4. Relative contributions of the three canonical variables, calculated for the discriminant functions analyses based on the carapace variables for the four genetically defined population groups.

Canonical Variable	Cumulative Percentage	Eigen Value
Canonical variable 1	59.62	1.276
Canonical variable 2	90.03	0.903
Canonical variable 3	100.00	0.206

Table 3. 5. Percent correct a posteriori classification to groups based on the morphometric classification function of the carapace variables for the four genetically defined population groups.

	Population Group				% Correctly
Group	A	B	C	D	Classified
Group A	81	2	13	1	83.50
Group B	10	71	17	1	71.71
Group C	8	0	90	10	83.33
Group D	2	0	13	20	57.14

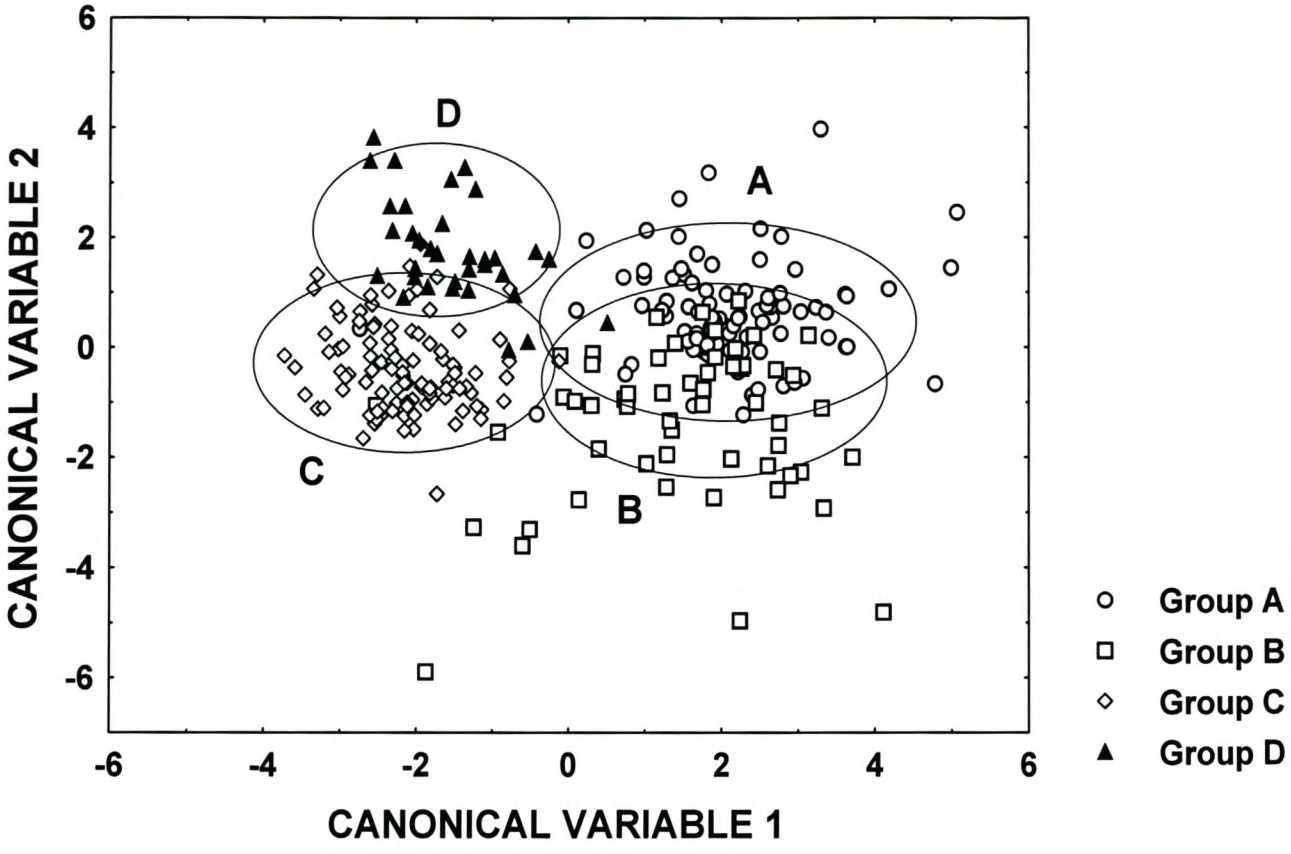


Figure 3. 5. Polygons encompassing the individual scores for the four genetically distinct population groups based on the log transformed pereopod variables. Group A (Cape Peninsula group); Group B (Intermediate group); Group C (Hottentots Holland group) and Group D (Fernkloof population).

The first two canonical variables contributed 97.54 % to the total variation between groups (table 3. 6). The classification functions for the four population groups showed a moderate degree of differentiation (table 3. 7).

The slopes of the regression of carapace width anteriorly (CWA) against carapace length (CL) were significantly different, (F= 152.46; $P < 0.01$) with group C (*P. brincki*) being broader than specimens of the same length of group A (fig. 3. 6a). The slopes of the regressions for the carapace height (CH) against carapace length (CL) for the two taxa could be discerned statistically (F = 110.07; $P < 0.01$), with *P. brincki* being proportionally deeper bodied than group A (fig. 3. 6b). However, group A was proportionally broader in its carapace width posteriorly (CWP) (F = 123. 82; $P < 0.01$) and in the distance between the medial margins of the orbits (fig. 3. 7a) (ED) (F = 11.84; $P < 0.01$) relative to CL when compared to group C (*P. brincki*) (fig. 3. 7b). When the slope of the carapace widest width (CWW) was compared between the two taxa relative to CL the slope was significantly different (F = 39.12; $P < 0.01$). Group C (*P. brincki*) specimens are wider in CWW relative to specimens of a similar length in group A (fig. 3. 8a). The distance between the postfrontal crest and the anterior margin of the carapace (PFCD) was plotted against the carapace length (CL) and compared between the two groups (*P. brincki* and group A). No significant differences in the slopes of these regressions could be detected (F = 0.010; $P > 0.05$). The slopes of the regression for the width of the merus of pereopod 2 (P2MW) regressed over the length of the merus of pereopod 2 (P2ML) were statistically different (F = 23. 89; $P < 0.01$), with group C specimens being broader than specimens of group A (fig. 3. 8b).

Table 3. 6. Relative contributions of the three canonical variables, calculated for the discriminant functions analyses based on the pereopod variables for the four genetically defined population groups.

Canonical Variable	Cumulative Percentage	Eigen Value
Canonical variable 1	78.54	3.856
Canonical variable 2	97.55	0.933
Canonical variable 3	100	0.120

Table 3. 7. Percent correct a posteriori classification to groups based on the morphometric classification function of the pereopod variables for the four genetically defined population groups.

	Population Group				% Correctly
Group	A	B	C	D	Classified
Group A	80	7	2	1	88.88
Group B	14	38	4	0	67.85
Group C	0	0	100	3	97.08
Group D	0	1	5	28	82.35

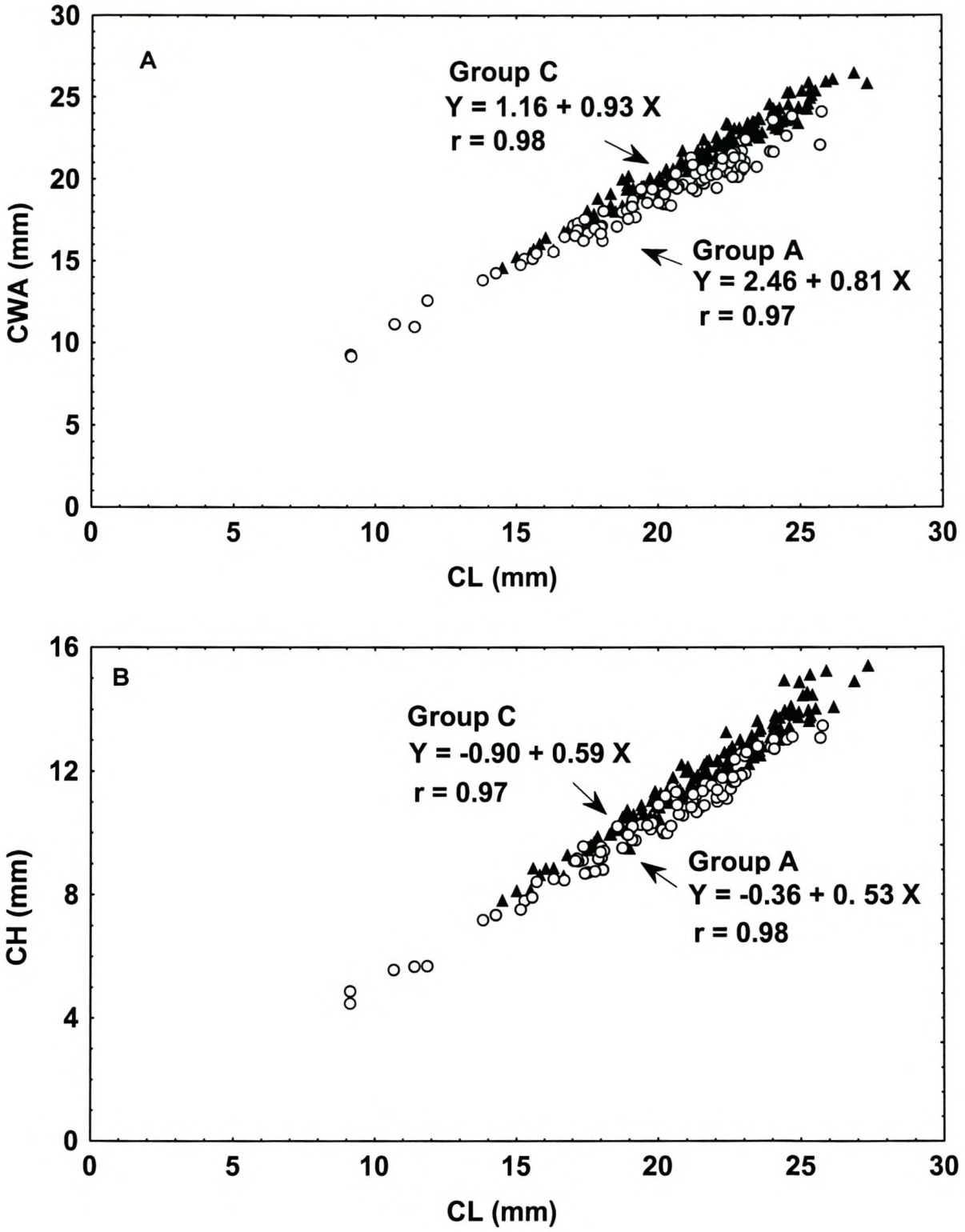


Figure 3. 6. Comparison of the regression of (A) CWA (carapace width anterior) over CL (carapace length) and (B) CH (carapace depth) over CL between group A and C (*P. brincki*).

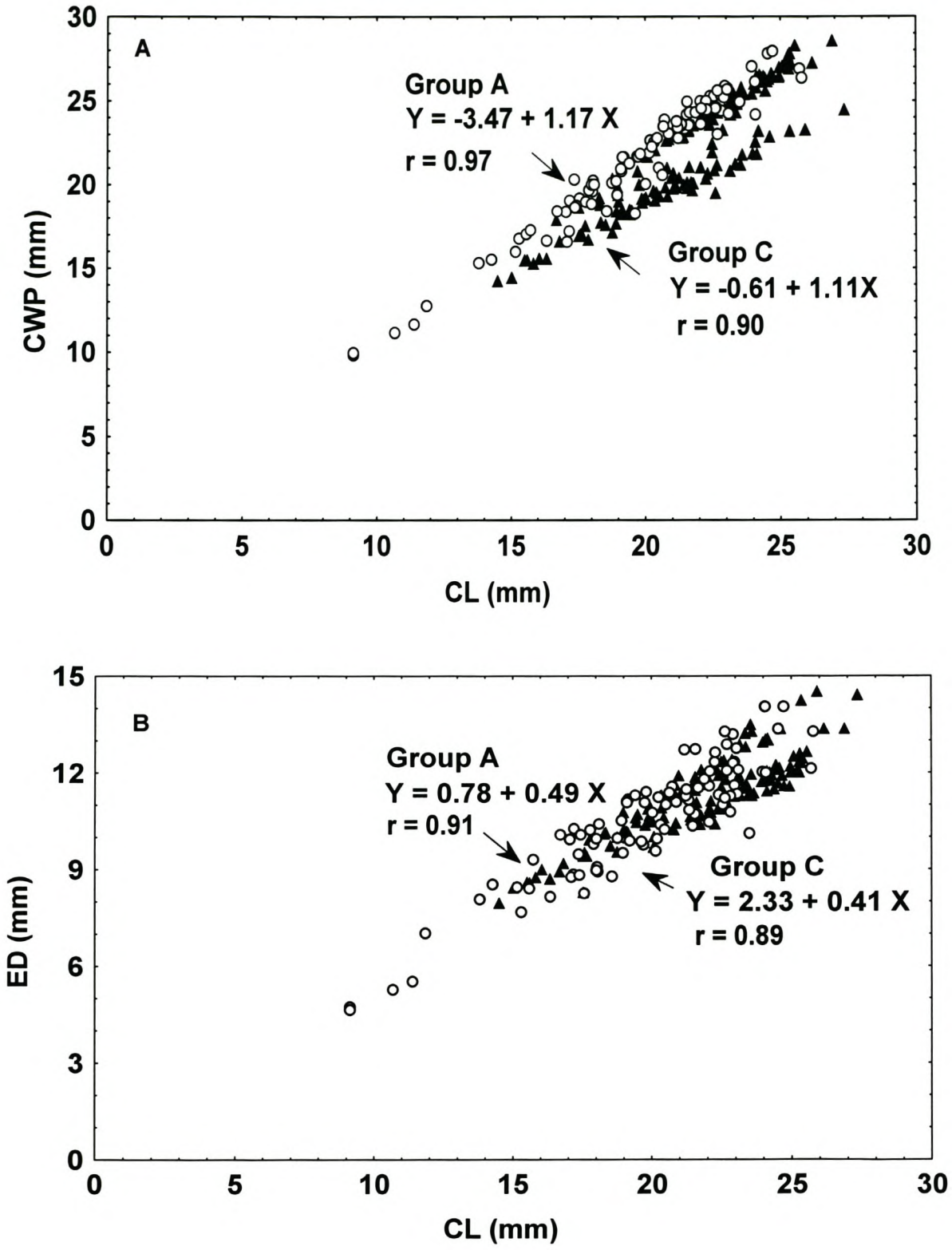


Figure 3. 7. Comparison of the regression of (A) CWP (carapace width posterior) over CL and (B) ED (orbital distance) over CL between group A and C (*P. brincki*).

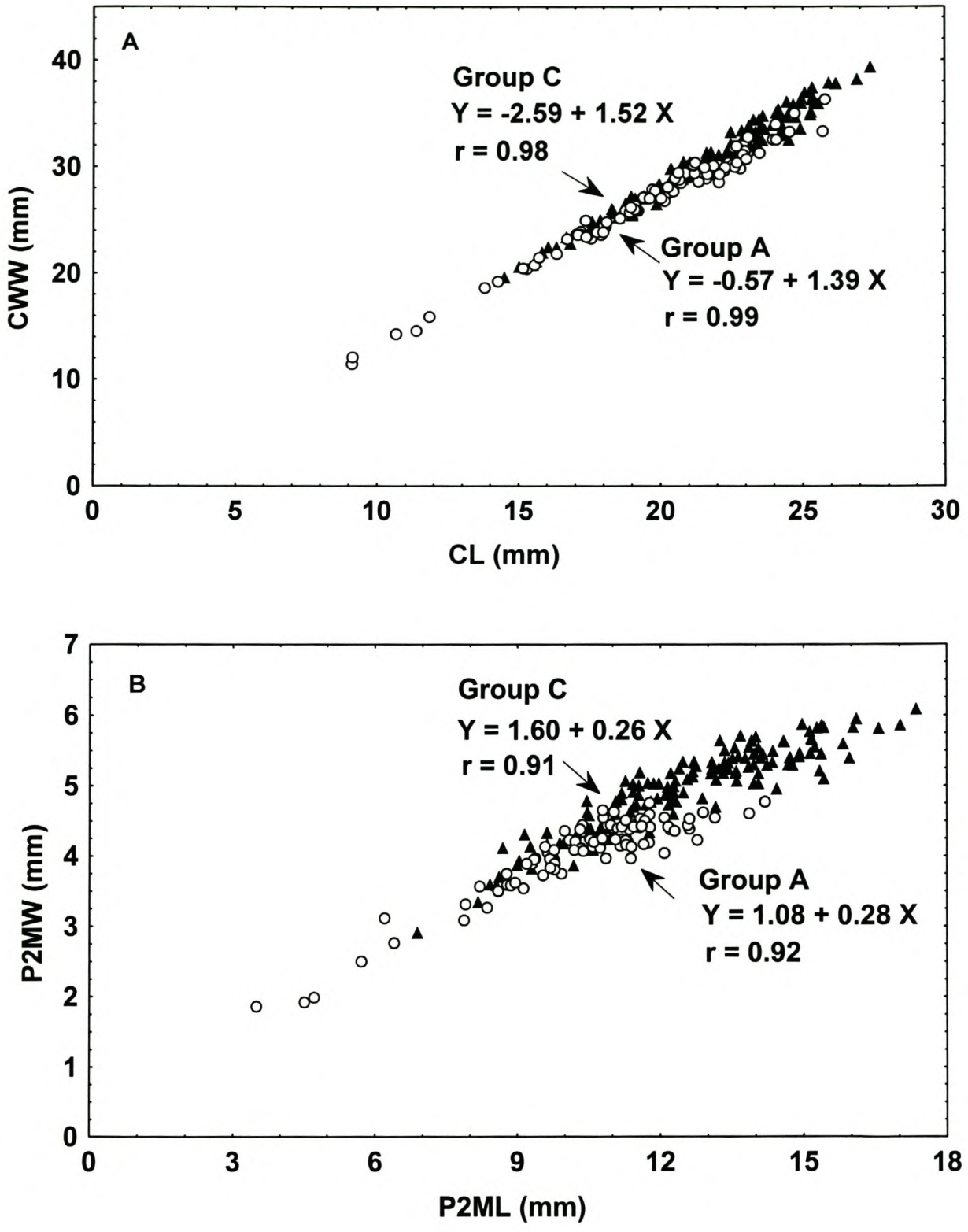


Figure 3. 8. Comparison of the regression of (A) CWW (carapace widest width) over CL and (B); P2MW (pereopod 2 merus width) over P2ML (pereopod 2 merus length) between group A and C (*P. brincki*).

The width of the propodus of pereopod 2 (P2PW) was compared against the length of the propodus of pereopod 2 (P2PL), and were found to be statistically significant (F = 216.44; $P < 0.01$) with group A being proportionally larger, than group C (*P. brincki*) specimens of a similar size (fig. 3. 9a). The slopes of the regressions for the merus width of pereopod 5 (P5MW) were compared to the merus length of pereopod 5 (P5ML) and found to be statistically significant (F = 6.52; $P < 0$ .05) with group C (*P. brincki*) specimens being larger than in group A (fig. 3. 9b). When the width of the propodus of pereopod 5 (P5PW) (F = 108.76; $P < 0.01$) was compared against the length of the propodus of pereopod 5 (P5PL), group A specimens were proportionally larger then specimens of group C (*P.brincki*) (fig. 3. 10).

Qualitative analyses

Mandibular palp

All specimens falling into groups A and B had similar mandibular palps, with the terminal segment bearing a dense tuft of setae on the proximal third of the segment. There is no ridge on the terminal segment. However, in the Hottentot's Holland group (group C) and Fernkloof (group D) the terminal segment consistently contains a dense tuft of setae arising from a flange or a ridge on the proximal third of the segment (fig. 3. 11).

Pleopod (gonopod) 1

The structure of pleopod 1 was similar in the Cape Peninsula group (group A) and the intermediate group (group B). Pleopod 1 was characterized by a smooth terminal segment curving away at the midpoint when viewed posteriorly, widest at base, ending in a pointed tip that curves slightly.

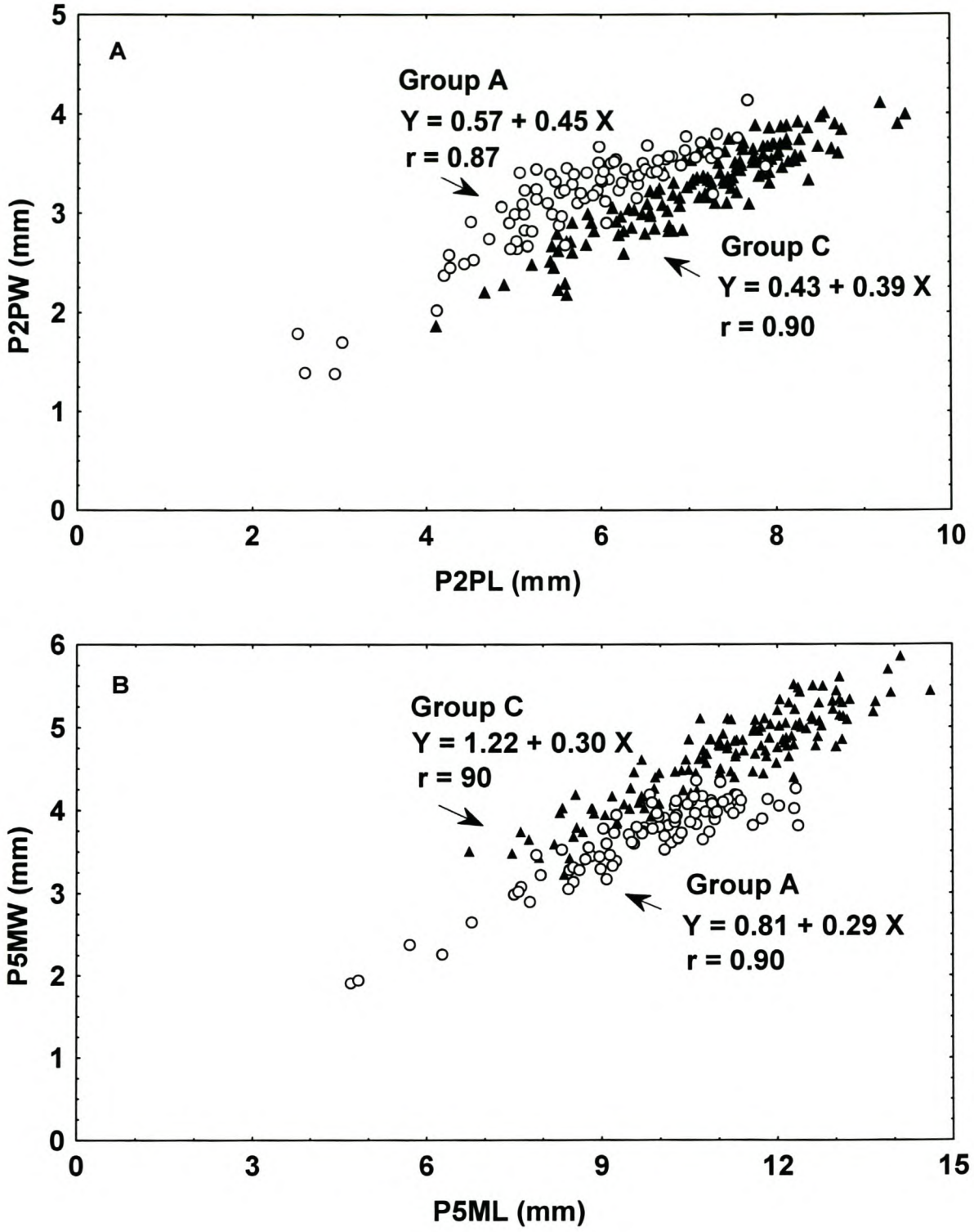


Figure 3. 9. Comparison of the regression of (A) P2PW (pereopod 2 propodus length) over P2PL (pereopod 2 propodus length) and (B) P5MW (pereopod 5 merus width) over P5ML (pereopod 5 merus length) between group A and C (*P. brincki*).

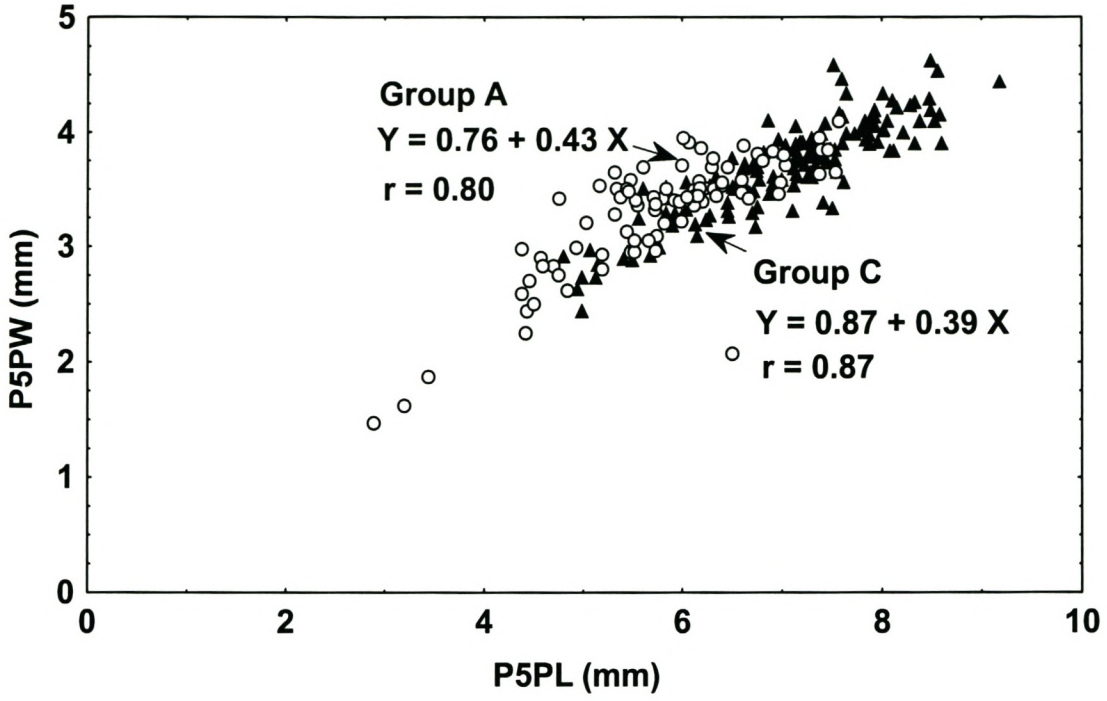


Figure 3. 10. Comparison of the regression of P5PW (pereopod 5, propodus width) over P5PL (pereopod 5, propodus length) between group A and C (*P. brincki*).

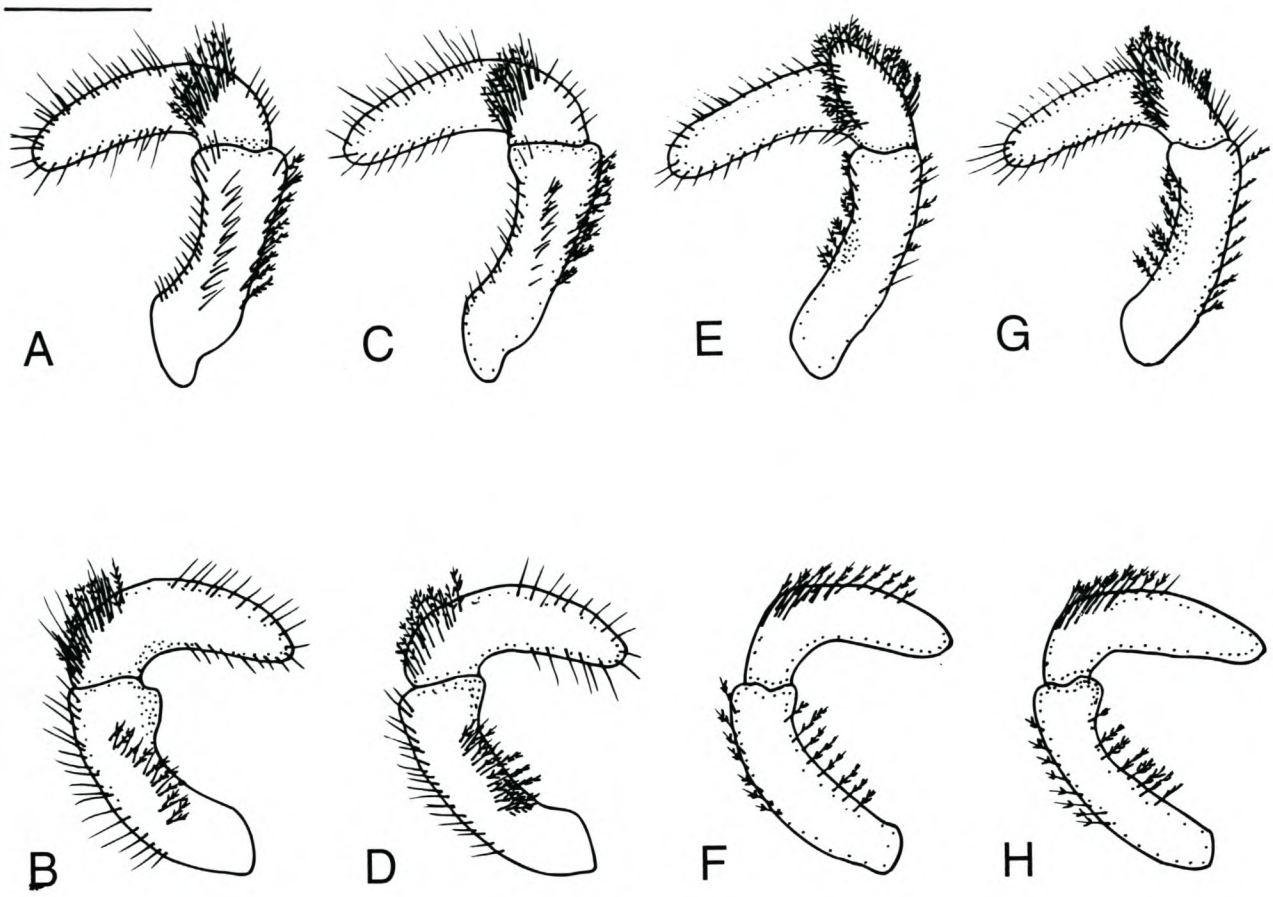


Figure 3. 11. Left mandibular palp. Blinkwater, A, posterior view, B anterior view; Jonkershoek, C, posterior view, D, anterior view; Rooiels, E, posterior view, F, anterior view and Fernkloof, G, posterior view, H, anterior view. Scale line represents 10mm.

The terminal segment was more strongly curved in groups A and B than in groups C and D (fig. 3. 12). In addition, the inner lateral margin of the subterminal segment pleopod 1 was markedly irregular in groups A and B, while it was only slightly irregular in groups C and D.

Discussion

Congruent patterns of genetic and morphological data strongly suggest the presence of two distinct species. Not only do these two main groups A (Cape Peninsula) and C (Hottentots Holland) separate at a genetic *I* of 0.73, but their genetic distinctiveness is further corroborated by the high mean $F_{(ST)}$ values. The presence of fixed allelic differences at three loci (*Ark-1, Gpi-1* and *Me-1*) between groups A and C indicate that they are genetically isolated, with no gene flow occurring presently. Furthermore, the presence of a distinct flange on the proximal third of the terminal segment of the mandibular palp in one of the putative species and the occurrence of considerable differences in the structure of pleopod 1 between the two putative species provides further supports for the recognition and delimitation of a species boundary. Gouldstein *et al.,* (2000) argue that the delineation of species boundaries should be robust and include as many fixed characters as are available. This is clearly the case in the present study, with both the genetic and morphological data, supporting the presence of two distinct species.

Populations collected from the geographically intermediate group B (Jonkershoek, Lourens River, Sir Lowry's Pass) were genetically ($I = 0.88$), and morphologically similar, to group A (Cape Peninsula). This group lacked the flange on the terminal

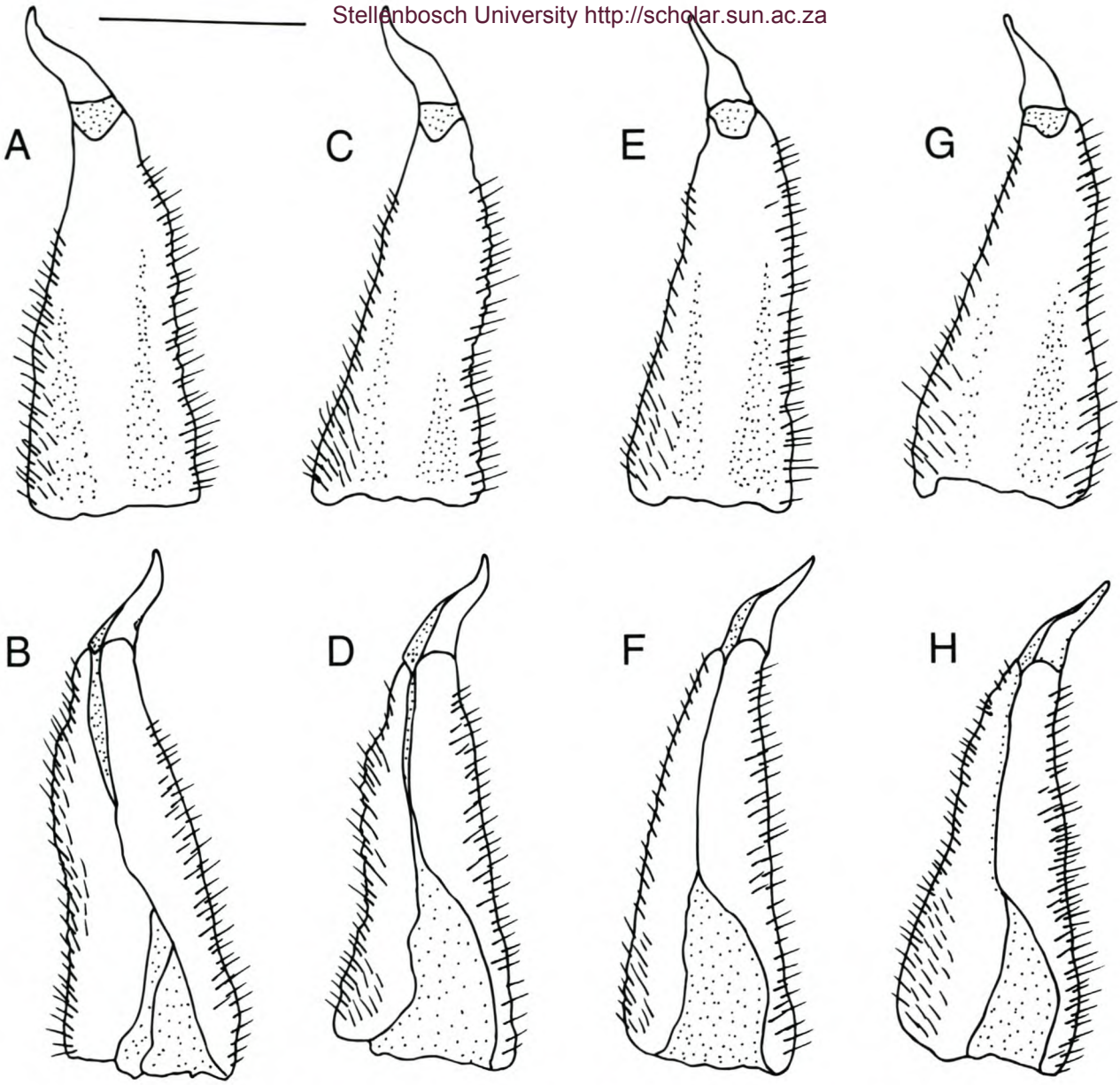


Figure 3. 12. Left pleopod, 1. Blinkwater, A, posterior view, B anterior view; Jonkershoek, C, posterior view, D, anterior view; Rooiels, E, posterior view, F, anterior view and Fernkloof, G, posterior view, H, anterior view. Scale line represents 10mm.

segment of the mandibular palp, and had a very irregular margin on pleopod 1. However, groups A and B were fixed for an alternative allele at *Ark-1*. Group C populations (Hottentots Holland) were genetically (I = 0.91) and morphologically similar to group D (Fernkloof) with all specimens possessing the distinct flange or ridge on the terminal segment of the mandibular palp, and having a relatively smooth inner margin on pleopod 1. A fixed allelic difference at *Ark-1*, is also present between groups C and D.

Interspecific genetic identity values obtained for freshwater crabs studied thus far have generally been less than < 0.85. The *I* value obtained in the current study (*I* = 0.73) falls within the range reported for congeneric taxa. For example, the genetic *I* between *P. perlatus* (H. Milne-Edwards, 1837) and *P. parvispina* (Stewart 1997b) was at 0.68 (Stewart 1997b), between *P. unispinus* (Stewart and Cook, 1998) and *P. sidneyi* at 0.66 (Stewart and Cook 1998), between *P. sidneyi* and *P.lividus* (Gouws *et al.,* 2001) at 0.82 (Gouws *et al.,* 2000) and between *P. depressus* (Krauss, 1843) and *P. clarus* (Gouws *et al.*, 2000) at 0.59 (Gouws, 1999). Suzuki and Okano (2000) recently reported that three freshwater crab species in the genus *Geothelphusa* (Stimpson, 1859) could also be separated at a genetic *I* < 0.85. Similar results have been reported for other marine decapod crustaceans. For example, Bert (1986) reported a genetic *I* of 0.46 for two allopatric stone crab species of the genus *Menippe.* Sbordoni *et al.,* (1990) reported a mean *I* value of 0.79 for two genetically diverged populations of the cave dwelling shrimp *Troglocaris anophthalmus* (Kollar, 1848), while Thompson (1996) reported an *I* value of 0.759 for two species of rock lobster of the genus *Panulirus.* Thorpe and Sole-Cava (1994) showed that congeneric

invertebrate taxa have *I* values that range between 0.34 to 0.85. The high $F_{(ST)}$ value reported in this study indicates that there is substantial genetic differentiation between the 11 populations examined. The high mean of 0.838 indicates that 83.8 % of the total genetic variation results from differences between populations, while 16.2 % results from variation within populations. Mean interspecific $F_{(ST)}$ values reported between freshwater crabs species, generally exceeds 0.60. For example, the mean $F_{(ST)}$ value between populations of *P. perlatus* and *P. granularis* was 0.645 (Daniels *et al.,* 1999); while between *P. sidneyi* and *P. perlatus* it was 0.672 (Gouws, 1999); between *P. sidneyi* and *P. lividus* it was 0.645 (unpublished data) and between *P. depressus* and *P. clarus* it was 0.872 (unpublished data). The mean $F_{(ST)}$ obtained between the Cape Peninsula and the Hottentot's Holland populations clearly falls within the range reported for congeneric taxa.

Intraspecific levels of genetic differentiation for freshwater crabs are generally low and have been found to be > 0.85 (Daniels *et al.,* 1998; 1999a, b). Conspecific populations generally show slight allele frequency differences at a few loci, while congeneric taxa are fixed for alternative alleles at certain loci. Limited genetic variation as derived from the genetic *I* values are evident within each of the four groups. However, $F_{(ST)}$ values within each of the four groups showed that some genetic structuring was present. In group A, a moderate degree of genetic structuring was evident, while in group B and C limited genetic structuring was observed. $F_{(ST)}$ values between catchments for three mountain stream river crabs were at 0.032, 0.085 and 0.127 for *P. parvispina, P. clarus* and *P. depressus*, respectively (Daniels *et al.,* 1998; unpublished data). These results indicate that genetic structure in mountain

stream taxa may be moderate to high. Hughes *et al.,* (1995) reported that the $F_{(ST)}$ values vary in the freshwater shrimp *Paratya austarliensis* and are largely dependent on the spatial level examined, with populations within streams having an $F_{(ST)}$ of 0.06 while those from different catchments have a mean of 0.57. The large $F_{(ST)}$ values obtained in the present study suggest that limited dispersal is occurring between populations. More recently, Wishart and Hughes (2001) reported that populations of the net-winged midge, *Elporia barnardi*, distributed on Table Mountain had a mean $F_{(ST)}$ of 0.24, while those at Jonkershoek had a mean of 0.02, with the total variation between the two groups being at 0.39. These results are particularly interesting because the freshwater crabs examined from these two areas, Table Mountain and the Jonkershoek showed very similar patters of genetic differentiation, with a high mean $F_{(ST)}$.

Generally, intraspecific $F_{(ST)}$ values have been reported to be low for crustaceans. For example, Creasey *et al.*, (1997) reported that in the majid spider crab, *Encephaloides armstrongi* (Wood Mason, 1891) the mean $F_{(ST)}$ was at 0.005. Similarly, two studies on the giant tiger prawn, *Penaeus monodon* (Fabricus, 1798) by Benzie *et al.,* (1992) and Forbes *et al.,* (1999) reported a mean value of 0.031and 0.007 respectively. Passamonti *et al.,* (1997) reported a mean $F_{(ST)}$ of 0.059 for the Norwegian lobster, *Nephrops norvegicus* (L). These results are not surprising considering that all the above mentioned decapods are marine species, and the marine environment is generally more continuous as opposed to freshwater environments.

Heterozygosity values for the freshwater crabs were low within each of the groups,

and ranged from 0.002 in group C, to 0.009 in group B, to 0.020 in group D to 0.037 in group A. Low heterozygosity values are well documented amongst potamonautid crabs (Daniels *et al.,* 1998, 1999a, b) and are generally below the mean of 0.048 calculated for decapods in general (Hedgecock *et al.*, 1982).

The question now arises as to whether these two groups A and C are sufficiently diverged to be two separate species. When species are in sympatry, a single fixed locus can indicate that interbreeding is not occurring. Richardson *et al.,* (1986) suggest that allopatric populations fixed at more that 20 % of all loci can be regarded with confidence as separate biological species. In the present study, 43 % (3 of 7) of all loci examined between group A and C were fixed, thus supporting the idea that group A is a new and as yet undescribed species. Defining populations that are in allopatry as distinct species is often met with difficulty, as the potential for gene exchange (reproduction) can not truly be assessed. The Biological Species Concept defines a species as "a group of interbreeding or potentially interbreeding populations, reproductively, or genetically isolated from other such groups'' (Mayr, 1964). Bock (1992) identifies three major properties, which are possessed by fully evolved species, namely, genetic, reproductive, and ecological isolation. He further argues that some species may not have all these properties totally developed, and that it not always necessary to demonstrate reproductive isolation for all species. Groups A and C are genetically isolated from each other, thus complying with one of the criteria as proposed by Mayr (1964) and should thus be considered distinct species. Group B is genetically conspecific to group A, and morphologically similar to this group, while group D is also conspecific to group C and morphologically similar to this group.

These results may thus indicate that groups B and D may have diverged recently from groups A and C, respectively, as only a single fixed allelic difference exists between these population groups, indicating a lack of contemporary gene flow. The phylogenetic species concept defines a species as " the smallest diagnosable cluster of individual organisms with which there is a parental pattern of descent" (Cracraft, 1989). Arguably, the flange or ridge on the terminal segment of the mandibular palp represents such a diagnostic heritable trait. However, the monophyly of this trait is unknown. Considering the congruence between the genetic and morphological data sets, these distinct allopatric lineages, should best be regarded as separate species (Sites and Crandall 1997).

Spatial patterns of genetic variation are at present not environmentally dependent and are thus likely to reflect historical and contemporary factors that affected the population structure (Riddle, 1996). Using the genetic *I* values, and two molecular clocks for allozyme data (Nei and Roychoundhury 1974; Yang *et al.,* 1974) the time of divergence between groups A and C falls between the Pliocene / Miocene, while the divergence between group A and B and C and D falls between the Pleistocene / late Pliocene. Climatological and geological changes that have occurred during this time period are likely to have resulted in the contraction and expansion of inland taxa. Throughout the Miocene, the climate oscillated between warmer and cooler phases, associated with eustatically-induced transgressions (higher sea levels) and regressions (lower sea levels) of sea level respectively. Sea level changes altered the climate, mainly affecting rainfall and temperature. Transgressions were typically associated with higher temperatures and increase in rainfall, regressions were characterized by

increase aridity and lower temperatures (Deacon, 1985). During the late Miocene climate became cooler and progressively more arid. These xeric climatic conditions were further aided by the development of the Benguela current. At the beginning of the Pliocene, sea levels started to rise again, although not as high as in the Miocene. By the late Pliocene, sea levels were more or less similar to those in the Pleistocene (Tyson, 1986). Groups A and C may have speciated during the regression that occurred at the end of the late Miocene / early Pliocene. Subsequent changes in sea level and climate during the late Pliocene / early Pleistocene resulted in more arid conditions and led to the fragmentation between groups A and B and between groups C and D.

The endemic aquatic invertebrates in the Western Cape typically occupy palaeogenic zones (upper-reaches of forest streams, riverine forest and caves). Evidence is emerging that suggests that these high mountain stream invertebrates are highly specious, and that each mountain possesses a unique biota. Studies on the population genetic structure of mountain stream invertebrates has reported considerable genetic divergence between taxa from the Cape Peninsula Mountains and those of the Hottentot's Holland Mountains. The consistent west / east split suggests that an environmental barrier(s) may exist that prevents gene flow between mountain ranges. For example, the data from the present study, as well as data on the freshwater amphipod genus *Paramelita* (Schellenberg, 1926) and studies on the, net-winged midge species, *Elporia barnardi*, and recent work on the freshwater isopod genus, *Mesamphisopus* (Nicholls, 1943) supports the hypothesis that at least two distinct biogeographic areas exist on mountain chains in the Western Cape (Stewart, 1992a,b;

Wishart and Hughes, 2001; Gouws, pers. comm). These results support the hypothesis that considerable speciation has occurred between the freshwater mountain stream species that occur on Cape Peninsula and those from Hottentot's Holland Mountains. Although the effect of climatological and geological change had a profound effect on the population genetic structure and distribution of these aquatic organisms, the fact that the three freshwater crustaceans (*Mesamphisopus capensis*, *P. brincki* and *Paramelita*) so far examined are direct developers may further have contributed to the pattern of genetic differentiation that has been observed.

Morphometrically and morphologically, groups A and C are distinct, as evident in the structure of the mandibular palp and the consistent difference in the structure of pleopod 1. Morphometrically the two main population groups can be distinguished easily, which corroborates the genetic distinctiveness of group A and C. As *P. brincki* was described from a population collected on the Hottentot's Holland Mountains (group C), the Cape Peninsula form, group A is thus a new species, the description of which follows.

Description of taxa

Potamonautes parvicorpus sp. n.

Holotype. One male, South Africa, Liesbeek river, 30 January 1990, collected by M. Hill (SAM A 44166).Paratypes. Same data as for holotype, five males and four females (SAM A 41141). Other material. Blinkwater Ravine, three males and three females, 3 May 1993, collected by B. A. Stewart and A. Mader, (SAM A 41136); Blinkwater Ravine, one female, November 1971, anonymous collector, (SAM A 41190); Orange Kloof, Table Mountain, five males, five females, 28 September 1994,

collected by J. Hulley (SAM A 41195); Noordhoek, De Goede Hoop river, two males and four females, 26 July 1996, A. Mader, (SAM A 44167); Platteklip, Table Mountain, four males five females and one juvenile, November 1999, collected by M. Wishart, (SAM A 44172); Silvermine, one female, November 1999, collected by M. Wishart (SAM A 44168); Vishoek, five juveniles, November 1999, collected by M. Wishart (SAM A 44171); Skeleton Gorge, two males, one female and four juveniles, September, 1999, collected by M. Wishart, (SAM A 44173); Disa Stream, one male, two females and two juveniles, September, 1999, collected by M. Wishart, (SAM A 44169).

Type locality. Collected from the Liesbeek River system Table Mountain, Cape Town, South Africa. This species is known from high mountain streams on the Cape Peninsular where it prefers unpolluted streams with leaf cover and small boulders.

Etymology. The specific epithet has been taken from the Latin, "parvus", meaning small, alluding to the small body of the animal, while the Latin, "corpus" refers to the body. The specific name hence is an adjective, agreeing in gender with the (masculine) generic name

Description

Male (holotype)

Measurements of the holotype are given in table 3.8. Carapace and limbs dark chocolate brown fading to a lighter shade of brown when preserved (fig. 3. 13,A-C). Caphalothorax ovoid, distinctly arched, maximum height and width at anterior third

Table 3. 8. Measurements (in mm) of the holotype and ranges of measurements for *Potamonautes parvicorpus* sp.n.

Variable	Abbreviation	Holotype	Males	Females
Carapace length	CL	18.36	24.08-11.85	24.71-9.13
Carapace widest width	CWW	25.31	36.24-15.9	34.99-11.41
Carapace width posteriorly	CWP	17.33	26.38-12.75	27.95-9.84
Carapace height	CH	10.02	13.48-5.68	13.12-4.47
Distance between postfrontal crest and the anterior margins of the carapace	PFCD	2.72	4.27-2.06	3.82-1.42
Distance between orbits	ED	10.07	13.28-7.03	14.07-4.73
Distance between exorbital teeth	CWA	11.57	24.09-12.57	23.84-9.27
Width of six abdominal segment	AW6	3.26	7.63-3.62	21.57-3.10
Major cheliped propodus length	MCPL	15.73	25.08-9.60	19.15-6.59
Major cheliped propodus height	MCPH	5.93	12.81-3.34	10.84-2.41
Pereopod 2, merus length	P2ML	5.94	14.20-6.21	12.62-1.92
Pereopod 2, merus width	P2MW	2.92	4.77-2.76	4.44-1.92

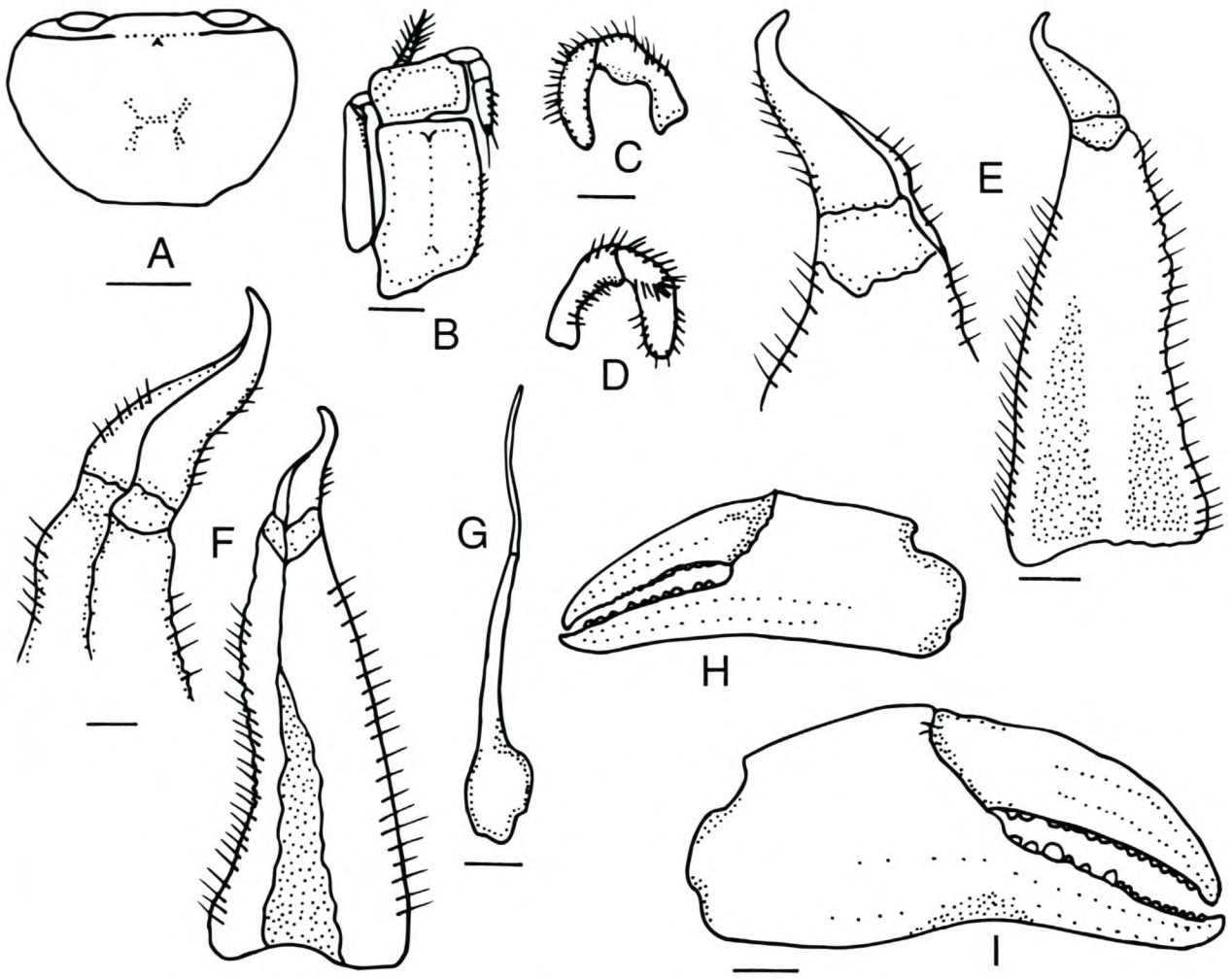


Figure 3. 14. *Potamonautes parvicorpus* sp. n. Male. Holotype. A, carapace outline. B, right third maxilliped. C. right mandibular palp, anterior view. D, right mandibular palp, posterior view. E, right pleopod 1, posterior view. F, right pelopod 1 anterior view. G, right pleopod 2, anterior view. H, left cheliped, dactylus and propodus. I, right cheliped, dactylus and propodus. Scale bars for the chelipeds and carapace= 10 mm. The scale bar for the remaing features are = 1mm.

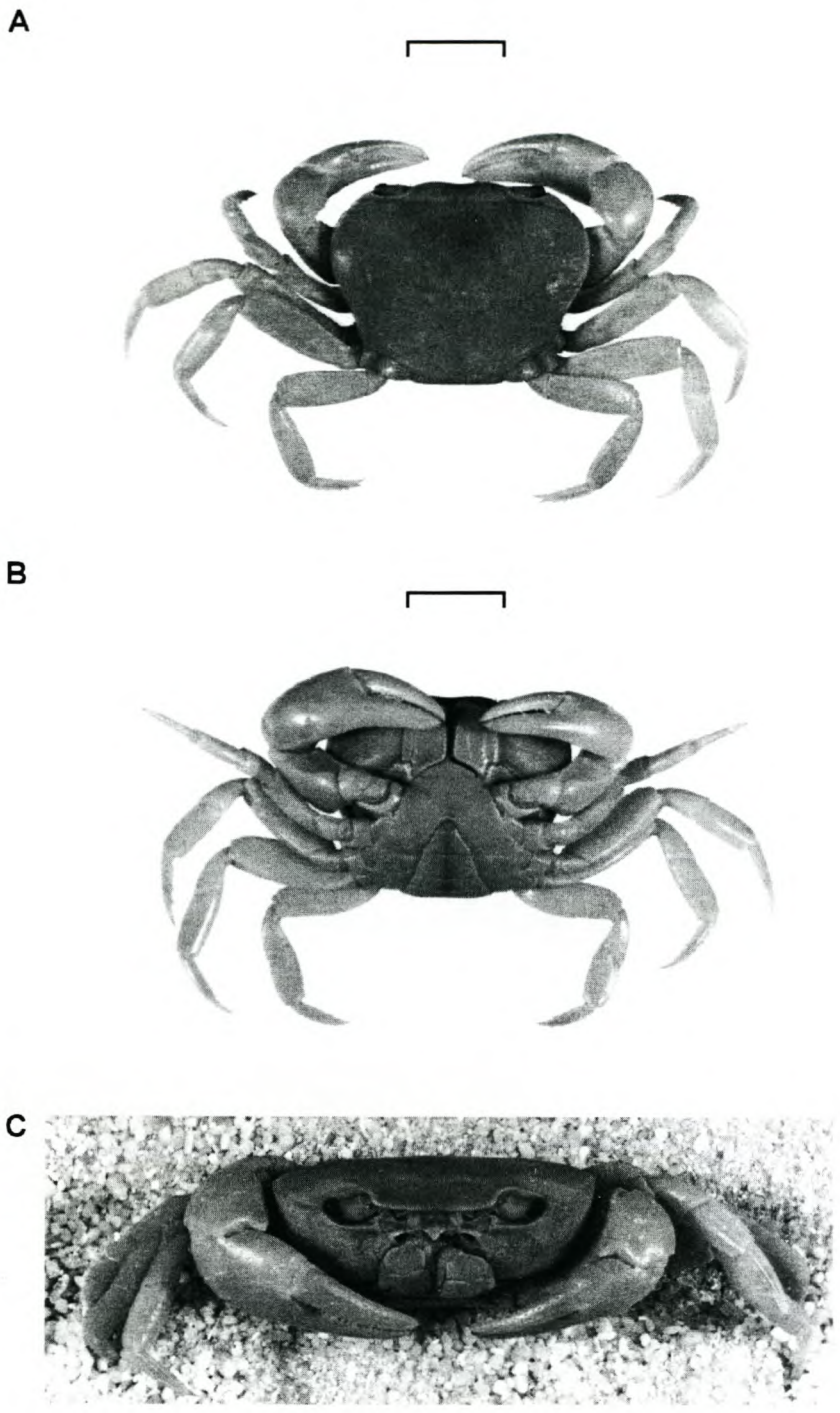


Figure 3. 13. *Potamonautes parvicorpus* sp.n. Male, Holotype, Liesbeek River, Cape Peninsula, South Africa, (CL = 18.36 mm) (SAM A 44166). A, whole animal, dorsal aspect; B whole animal, ventral aspect; C, cephalothorax, frontal aspect. Scale bar = 10 mm

(ratio CH / CL = 0.54, CWW / CL = 1.38) (fig. 3.13 A). Anterior margin of front slightly indented. Carapace smooth, urogastric and cardiac groove moderately deep. Exorbital teeth blunt, epibranchial teeth absent. Anterolateral margin between exorbital teeth and postfrontal crest finely granulated, lacking teeth. Anterolateral margins posterior to postfrontal crest finely granulated, margin not curving inward over the surface of carapace in branchial region. Postfrontal crest poorly developed posterior to eye orbits fading at midpoint, with a short-forked groove at midpoint. Each flank with longitudinal groove. Sternites 1 and 2 fused, no suture present. Suture between sternites 2 and 3 complete, deep suture between 3 and 4 complete, shallow lateral part sloping sharply towards abdomen. The third maxilliped fills the buccal frame except for a small oval efferent respiratory opening (fig. 3. 14 B). Flagellum present on exopod; ischium smooth, with faint vertical groove.

Mandibular palp with two segments, terminal segment undivided, with an extremely dense tuft of pinnate setae on the proximal third of the segment (fig. 3. 14 C and D). Chelipeds markedly unequal, dactylus of right cheliped slightly arched, both dactyli armed with several small to medium cutting teeth (fig. 3. 14 H and I). Propodus of right cheliped more swollen then the left, pollex armed with small cutting teeth. Carpus of both chelipeds with one large prominent spine and two rudimentary teeth, antero-infero margin of merus with no spine. Pereopodus slender, pereopods armed with sharp point, margins of dactyli of P2 - P5 smooth. Pleopod 1 (gonopod 1), terminal segment short, 0.25 length of the subterminal segment, terminal segment curving away from the midline when viewed posteriorly, widest at base, ending in a pointed tip (fig. 3. 14 E and F). Subterminal segment of pleopod 1 tapering distally,

inner lateral margin slightly irregular, posterior surface with twisting longtudinal groove, running length of both subterminal and terminal segments, anterior surface lacking longitudinal groove. Pleopod 2 (gonopod 2), hollow, about 0.65 length of subterminal segment, widest at base, tapering sharply inward at 0.4 times length, forming a narrow upright process supporting terminal segment (fig. 3. 14 G).

Variation. The anterolateral margins of juveniles may be beaded, but becomes smooth as the crab matures. Both sexes exhibit heterochely, with 86.84 % of males being right handed and 13,15 % being left handed, in females, 4 % were homochelic, 78.87 % right handed while 17 % were left handed.

Remarks. *P. parvicorpus* sp.n. bears superficial resemblance to other South African freshwater crabs. The new species can easily be distinguished from the six freshwater crab species with toothed epibranchial corners, these are *P. parvispina* (Stewart, 1997b), *P. dentatus* (Stewart, 1995), *P. warreni* (Calman, 1918), *P. unispinus* (Stewart and Cook, 1998) and *P. calcaratus* (Gordon, 1929). In *P. parvispina*, a small but distinct tooth is present on the epibranchial corner and this species is restricted to upper tributaries of the Berg and Olifants River systems. In *P. dentatus* the anterolateral margin is characterized by a series of teeth, and this species is endemic to KwaZulu- Natal. *P. warreni* is distinct from the new species as it possesses a single tooth or a series of well defined teeth on the anterolateral margins of the carapace, and is restricted to the Orange and Vaal River systems. *P. unispinus* is characterized by the presence of a single spine on the anterolateral margins of the carapace. In *P. obesus calcaratus* the anterolateral margin possesses a single spine like tooth. All

these species, with the exception of *P. parvispina* and *P. obesus calcaratus* occur in large river systems. *P. parvicorpus*_sp.n bears a number of similarities to *P. brincki* (Stewart, 1997a), however the new species does not have a flange on the terminal segment of the mandibular palp, and is genetically distinct from *P. brincki*. In addition the structure of pleopod 1 is markedly different between *P. brincki* and *P. parvicorpus* sp. n. In both *P. depressus* (Krauss, 1843) and *P. clarus* (Gouws *et al.,* 2000) the limbs are slender, and both are genetically distinct from the new species. *P. depressus* is brown to green-brown in color, while *P. clarus* is bright orange in color. *P.lividus* (Gouws *et al.,* 2001), a species restricted to swamp forests, is orange to red with a silver – blue sheen and is characterized by an inflated carapace, with highly arched chela. *P. perlatus* (H. Milne Edwards, 1837), *P. sidneyi* (Rathbun, 1904) and *P.granularis* (Daniels *et al*., 1998b) are all large species, where the postfrontal crest is well defined and beaded. These species generally live in larger river systems, and are often absent from high mountain streams.

Further afield, a number of freshwater crab species bears superficial resemblance to *P. parvicorpus* sp. nov. For example, *Potamonautes pilosus* (Hilgendorf, 1898) and *P. neumanni* (Hilgendorf, 1898) are restricted to East Africa, with *P. pilosus* possessing a depressed carapace while *P. neumanni* has a vaulted carapace. In *P. ballayi adentatus* (Bott, 1955) the postfrontal crest is complete and individuals possesses large gaping chela with slender fingers. The terminal segment of pleopod 1 in both *P. loveridgei* (Rathbun, 1933) and *P. macrobrachii* (Bott, 1953) is markedly different from that of *P. parvicorpus* sp. nov. *P. suprasulcatus* (Hilgendorf, 1898) possesses large, stout chelipeds and a finely granulated anterolateral margin. In both *P. walderi* (Colosi,

1924) and *P. bipartitus* (Hilgendorf, 1898) the medial invagination and postfrontal crest are concave.

The usefulness of the mandibular palp in freshwater crab systematics remains problematic as highlighted by the relationship between *P. parvicorpus* sp. nov and *P. brincki* (formerly *Gecarcinautes brincki*). According to Bott (1970a) the family Gecarcinucidae and the Potamonautidae can only be separated based on the terminal segment of the mandibular palp. In the Potamonautidae the palp is simple and undivided while in the Gecarcinucidae the palp is divided and bilobed. It was this character that led Bott (1965) to included *P. brincki* into the *Gecarcinautes*. The data from the present study demonstrated the existence of the bilobed mandibular palp in the Potamonautidae, casting doubt on its usefulness to distinguish these two families. Instead it would appear that a credible diagnostic feature that can be used successfully to distinguish the families are lacking or that the use of the bilobed mandibular palp needs refinment.

Bott (1965) also inferred that there is a close relationship between *G. brincki* and the Madagascan *Gecarcinautes*. The present study nullifies this hypothesis, however, it may possible that Potamonautidae and Gecarcinucoidea are indeed very closely allied. The findings from this tudy also casts doubt on the existence of genus *Seychellum* Ng and Takeda, 1994 (Gecarcinucidae: Gecarcinucoidea) as this new genus was errected based on the bilobed terminal segment. The utility of this character in diagnosing species and families needs radical re-assessment.

Chapter 4

Carapace dentition patterns, morphometrics and allozyme differentiation among two toothed freshwater crab species (*Potamonautes warreni* and *P. unispinus*) (Decapoda: Brachyura: Potamonautidae) from river systems in South Africa

Abstract

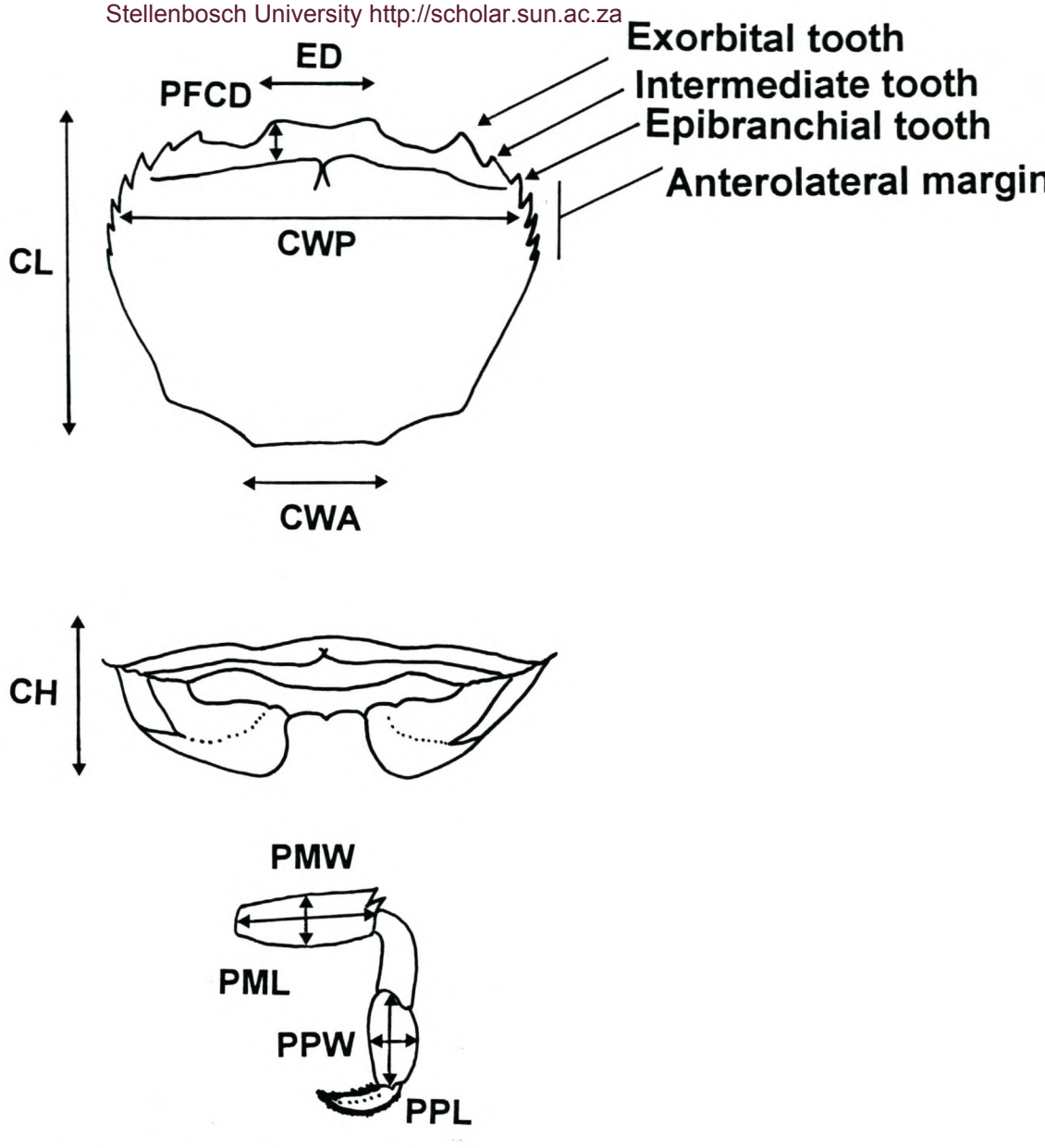
The taxonomic relationship between two toothed South African River crabs, *Potamonautes warreni* and *P. unispinus,* is unclear. The problem stems from the widespread variation in carapace dentition patterns amongst *P. warreni* individuals over its biogeographic range, where single toothed individuals may appear similar in carapace morphology to *P. unispinus*. Ten populations of *P. warreni* and 18 populations of *P. unispinus* were collected and the morphometric and genetic differentiation between the two taxa quantified. Patterns of morphometric and genetic variation were examined using multivariate statistics and protein gel electrophoresis. Principal component analyses of carapace characters showed that the two species are morphologically indistinguishable. However, discriminate functions analyses and additional statistical results corroborate the morphological distinction between the two taxa. Allozyme electrophoresis of 17 protein coding loci, indicated a close genetic similarity between the two species ($I = 0.92$). A fixed allelic difference at one locus (*Lt-2*) and extensive genetic variability at another locus (*Pgm-1*) indicate that two gene pools are present and that the two taxa are genetically isolated. Intraspecific genetic I values for both species were > 0.97 and indicated no apparent genetic structuring on a micro or macro-geographic scale. The variation in carapace dentition among *P. warreni* populations possesses no genetic basis and may likely be the

product of ecogenesis. Dentition patterns among river crab species appear to be conserved and reliable as species specific diagnostic markers, but should ideally be used in combination with other morphological data sets and genetic evidence.

Introduction

Species that are distributed over a broad geographic range may often display substantial morphological variability. When variation occurs in diagnostic morphological characters that are commonly used for species delineation, it becomes difficult or impossible for systematists to accurately discern species boundaries. The taxonomy of river crabs has long been plagued by considerable variability in diagnostic morphological features; particularly in the dentition pattern of the carapace margins. For example, the presence, or absence, and the size and shape of the epibranchial teeth to assign freshwater crabs to genera and subgenera (Alcock, 1910; Cumberlidge, 1999a).

In his study on the Indian freshwater crab fauna, Alcock (1909a, 1910) noted that the epibranchial tooth varied between species but generally provided a safe measure for delineating species (fig. 4.1). However, Alcock noted that the size of the intermediate teeth on the postfrontal crest between the exorbital tooth and the epibranchial tooth was fairly variable and less diagnostic between species. Bott (1955) used the dentition patterns of the epibranchial margins and intermediate tooth of the carapace margins to delineate families, genera, subgenera and species, and assigned considerable value to this morphological feature. More recently, Cumberlidge (1999a) remarked that although the size and shape of the epibranchial tooth may vary within genera, its presence is generally a conserved character that may be used in distinguishing genera and species amongst African freshwater crabs of the family Potamonautidae.



Key to legend

CWA = carapace widest width anterior
ED = orbital distance
PFCD = post frontal crest distance
CL = carapace length
CWP = carapace width posterior
CH = carapace height
PMW = pereopod merus width
PML = pereopod merus length
PPW = pereopod propodus width
PPL = pereopod propodus length

Figure 4. 1. Carapace outline showing the different dentition patterns in freshwater crabs and the morphometric measures taken.

In the systematics of river crab species dentition patterns have clearly been afforded considerable taxonomic value and remain a highly diagnostic morphological feature for species delineation. However, it is noteworthy that none of these authors (Bott, 1955; Cumberlidge, 1999a) have intensively examined intraspecific morphological variation in dentition patterns on a broad geographic scale.

The South African river crab fauna are well known to be characterised by considerable morphological variability (Barnard, 1935, 1950). *Potamonautes warreni* (Calman, 1918) is distinct from other river crabs as it typically has five to ten teeth on the anterolateral margins of the carapace. Calman (1918) remarked that this species might represent a morphological variety of *P. perlatus* (H. Milne Edwards, 1837). Due to the importance assigned to dentition patterns among freshwater crabs, *P. warreni* was retained as a species. Barnard (1935) reported that certain *P. warreni* specimens from populations in Southern Africa (Barberton, Lake Chrissie, Salisbury and Chishawasha) are characterised by fine and regularly decreasing serrations of the carapace margins. He noted that the dentition pattern in *P. warreni* varies from a "typical" form with five to ten well-defined teeth to specimens with a single tooth. He further remarked that some of the feebly dentate forms of *P. warreni* appear very similar to either *P. perlatus* or *P. sidneyi* (Rathbun, 1904) and were particularly difficult to separate. This led Barnard (1935) to suspect that these taxa may be hybridizing. He noted that the "typical strongly dentate form" of *P. warreni* occurs in the south and west while the "feebly dentate form" occur in the north and towards the east of South Africa. The single toothed form initially identified as a variety of *P. warreni* by Barnard (1935) was however recently described as a new species, *P.*

unispinus (Stewart and Cook, 1998). *P. unispinus* is distributed in the major river systems of the Northern Province and Mpumalanga in South Africa, but could be more widespread on the subcontinent, and *P. warreni* is restricted to the Orange River systems and its major tributaries in South Africa.

Potamonautes unispinus is distinct from *P. warreni* in possessing a single large tooth on the anterolateral margins of the carapace, and a beaded carapace margin. Stewart and Cook (1998) noted that geographically disjunct populations of these two species were genetically distinct based on allozymes. However, recent sampling of *P. warreni* populations from Gauteng, revealed individuals with five to ten well-defined teeth, and specimens with a single epibranchial tooth from the same locality. These single toothed specimens of *P. warreni* possibly resembled *P. unispinus.* When allozyme data for the morphologically variable specimens of *P. warreni* were compared with *P. unispinus,* preliminary results revealed limited genetic difference (Stewart and Ridgeway, unpubl. data). The validity of *P. unispinus* was thus in question. It remained unclear whether *P. unispinus* represented a separate species, or a morphological variety of *P. warreni,* or whether the extensive morphological variation in *P. warreni* populations could be attributed to hybridisation between these two taxa. The purpose of the present study is threefold. Firstly, to investigate the validity of the taxon *P. unispinus* with the aid of allozymes and morphometrics, secondly, to investigate intraspecific patterns in carapace dentition in *P. warreni*; and thirdly, to assess the systematic value of carapace dentition patterns in delineating river crab species in South Africa.

Among the methods that are commonly used in invertebrate systematics is allozyme electrophoresis (Thorpe and Sole Cava, 1994). The widespread use of allozymes has contributed significantly towards our understanding of genetic variation in natural populations, and has been used to conclude systematic debates (Grant *et al.*, 1988). The use of multivariate morphometrics in systematics has revitalised this field in recent years (Rohlf and Marcus, 1993). Morphometic studies have now become widely used in systematics and population studies among a wide range of organisms, including crustaceans (McClure and Wicksten, 1997; Overton *et al.*, 1997). A combination of allozymes and morphometrics is particularly powerful to determine population and species level variation. In the present study we used allozyme electrophoresis and multivariate morphometric analyses to quantify the degree of differentiation between the two taxa.

Materials and methods

Sample collection

Ten populations of *Potamonautes warreni* and 18 populations of *P. unispinus* were collected for genetic analyses along river systems (fig. 4. 2). Crabs were caught with handnets following their attraction to baited (ox heart) lines. On capture, crabs were killed by freezing at -20 °C for 24 h prior to measurement and tissue extraction at the field station.

Morphometrics analyses

The same measurements outlined in chapter three were taken for each specimen in the present study. The morphometrics matrix for statistical analyses was based on

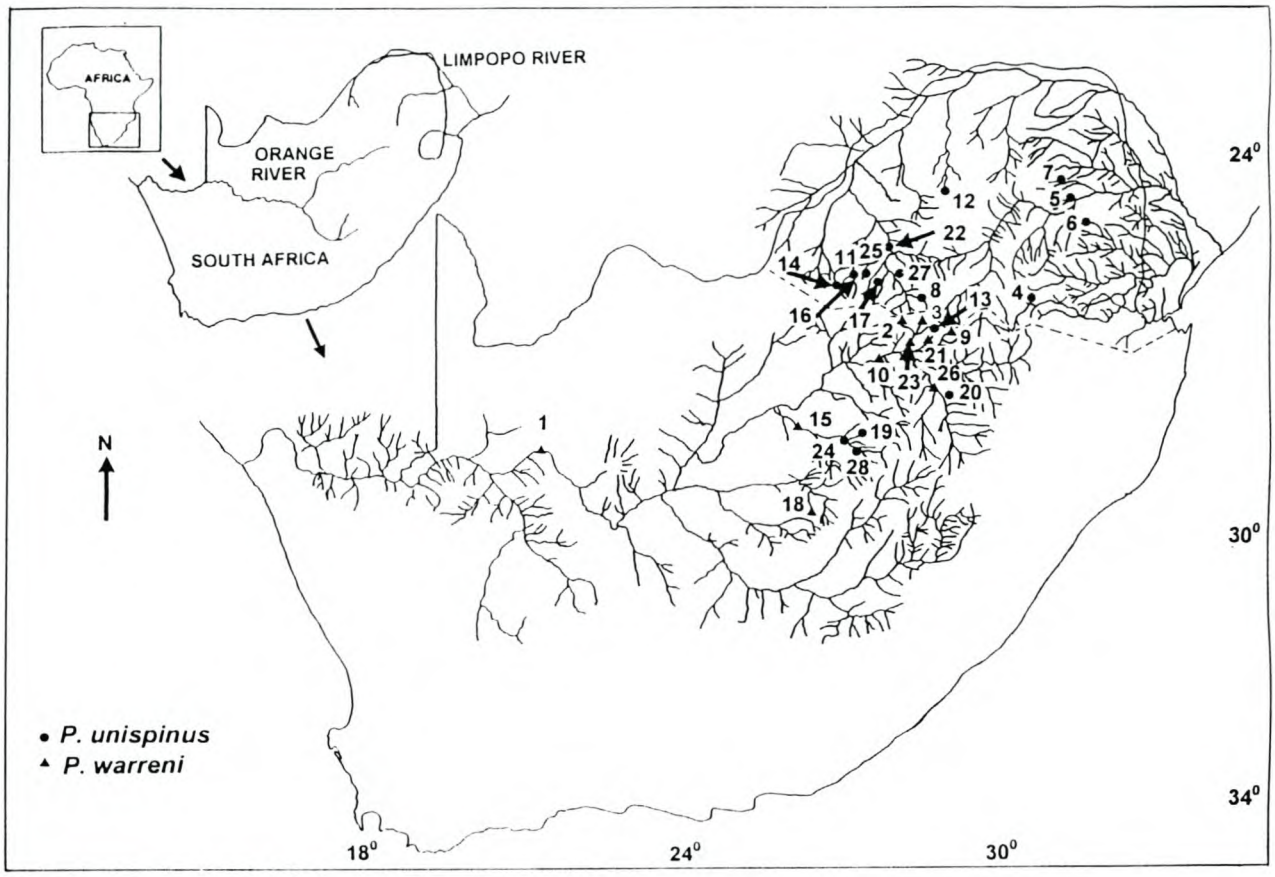


Figure 4. 2. Map showing the distribution of the populations sampled. *Potamonautes warreni* indicated by (▲) and *P. unispinus* by (●), (1) Upington, (2) Lenasea, (3) Olifants vlei, (4) Vaal B, (5) Shingwedzi, (6) Mbwedi, (7) Mutamba, (8) Edenvale, (9) Blesbokspruit, (10) Vereeniging, (11) Hex River, (12) Sand River, (13) Suikerbosrand Reserve, (14) Elands River, (15) Bothaville, (16) Sterkstroom, (17) Hartebeespoort dam, (18) Renosterspruit, (19) Vredespruit, (20) Frankfurt, (21) Suikerbosrand River, (22) Crocodile River, (23) Klip River, (24) Zout River, (25) Rustenburg Nature Reserve, (26) Villiers, (27) Pienaars River and (28) Willem Pretorius Game Park. The broken line indicates the boundary between the Limpopo River system and the Orange River system.

individual morphometric profiles of the body dimensions. Patterns of morphometric relationships can be influenced by the effect of size in species of undetermined age, and it may therefore be necessary to remove this component (McClure and Wicksten, 1997). Several methods are currently in use to overcome this problem. Principal Component Analyses (PCA) was used to investigate the relationships between the two species. These were carried out on the log transformed carapace data to assess differences between the populations of the two taxa. The factor scores were plotted on a two-dimensional plot. In addition, a discriminate functions analysis was performed on these log-transformed variables. The jack-knife method was then used to calculate the classification function and a discriminate function coefficient for each variable and each individual was calculated. Bivariate scatter plots were drawn for the most discriminatory carapace and non-carapace variables. The slopes of the bivariate plots were statistically compared using analyses of covariance (ANCOVA). These functions were performed in STATISTICA (StatSoft Inc, 1996).

Morphological Variation

Gonopod 1 morphology and the carapace outline of individuals of the two taxa were examined and drawn using a Wild stereo microscope attached to a camera lucida. The variation in the dentition patterns among *P. warreni* was examined by group. Group one comprised all the populations from the Northern Cape (Vioolsdrif, Upington, Gifkloof, Zak River and Fish River), while group two comprised populations collected within the Free State (Glen, Renostersprui t, Modder River, Bothaville, Kroonstad and Villiers) and group three comprised all the populations from Gauteng (Klip River, Lenasea, Olifantsvlei, Marievale, Vereeniging and Suikerbos Rand river)

and North West Province (Potchefstroom). The number of teeth on the left and right hand margins of the carapace were counted for each sample from the three population groups, and the carapace length for each sample was recorded. Analyses of variance (ANOVA) were used to test for differences in the mean number of teeth between the three population groups and to examine any patterns of geographic variation. These data were collected in order to assess whether single toothed *P. warreni* specimens occur over a wide geographic distribution or are confined to certain localities, or whether the variation in dentition exhibits a distinct pattern from the west to east coast. In addition, the mean number of teeth was counted in order to test whether there was a significant difference between males and females in the number of teeth on the left and right margins of the carapace using ANOVA. The mean number of teeth on the carapace margins in both sexes was plotted against the respective size class (CL). The distribution of the two taxa was mapped (based on collection records from the present study and specimens at in the South African Museum holdings).

Genetic analyses

The methods outlined in chapter three were adopted for this study. Briefly, genetic variation at 17 isozyme loci was examined on a 13 % starch gel, and electrophoresed using three buffer systems (table 4. 1). The numerical analyses were performed using the BIOSYS-1 programme (Swofford and Selander, 1981). Allelic and genotype frequencies were computed. Chi-square analyses were used to test if populations were in Hardy-Weinberg equilibrium. Levene's correction for small sample size was used. The mean heterozygosity (H) per locus for each population was calculated using Nei's (1978) unbiased estimates. The F statistics, including $F_{(IS)}$ (the mean value of genetic

Table 4. 1. Enzyme and buffer systems used during electrophoresis. N = the number of loci.

Enzyme	Abbreviation	Buffer	E.C.number	N
Arginine kinase	*Ark-1*	A	2.7.3.3	1
Glucose phosphate isomerase	*Gpi-1*	A	5.3.1.9	1
Glyceraldehyde-3-phosphate	*Gap-1*	C	1.2.1.12	1
Isocitric dehydrogenase	*Idh-1, 2*	B	1.1.1.42	2
Lactate dehydrogenase	*Ldh-1*	B	1.1.1.27	1
Peptidase (Glycyl leucine as substrate)	*Gl-1*	B	3.4.11-	1
Peptidase (Leucine tyrosine as substrate)	*Lt-1*	A	3.4.11-	2
Malate dehydrogenase	*Mdh-1, 2*	C	1.1.1.37	2
Malic enzyme	*Me-1*	C	1.1.1.40	1
Mannose phosphate isomerase	*Mpi-1*	C	5.3.1.8	1
Phosphoglucomutase	*Pgm-1, 2*	A	2.7.5.1	2
Phosphoglucosedehyrogenase	*Pgd-1*	A	1.1.1.44	1
Sorbitol dehydrogenase	*Sdh-1*	C	1.1.1.14	1

differentiation or inbreeding coefficient within subgroups), $F_{(IT)}$ (the mean value of genetic differentiation over the entire population) and $F_{(ST)}$ (the genetic differentiation between any two subpopulations), were calculated to determine the degree of genetic differentiation amongst the populations of the two taxa (Wright, 1965). The percentage of polymorphic loci in each population was determined. Loci were considered polymorphic if the frequency of the most common allele did not exceed 0.99. The mean unbiased genetic identity (*I*) among the populations were calculated from the allelic frequencies according to Nei (1978). These were used to construct a dendrogram of biochemical similarity using the UPGMA cluster algorithm.

Results

Morphometrics

The first two principal components extracted by the Principal Component Analyses contributed 96 % to the total variance between the two groups (table 4. 2). The weights of the first principal component (PC 1) were all positive and of similar magnitude suggesting that variation along this principal component is size related and thus not informative with respect to shape. Principal component 1 (PC1) was thus excluded from the graphic analyses. A plot of the second (PC 2) and third principal components (PC 3) revealed no distinct groups (fig. 4. 3). Similarly a plot of the PC 3 and fourth principal components (PC 4) also failed to show two distinct groupings (fig. 4. 4). For the second and fourth PC orbital distance (ED) contributed the most to the total variance, while for the third PC, the carapace widest width (CWW) contributed the most to the total variance. A discriminate functions analysis showed that the two groups could not be distinguished (fig. 4. 5).

Table 4. 2. Weights (eigen vectors) for the first four principal components for the Principal Components Analyses comparison for the two *Potamonautes* taxa.

PC 1, PC 2, PC 3 and PC 4 = first, second, third and fourth principal components.

	PC 1	PC 2	PC 3	PC 4
Eigen value	6.3365	0.4302	0.0865	0.0618
CL	0.9853	0.0107	0.1098	0.0192
CWW	0.9864	0.0184	0.1307	0.0471
CWP	0.8222	-0.5580	-0.0984	-0.0019
PFCD	0.9753	0.0146	-0.0575	-0.1134
ED	0.9453	0.2300	-0.1677	0.1589
CWA	0.9885	-0.0408	0.1138	0.0310
CH	0.9458	0.2522	-0.0572	-0.1419

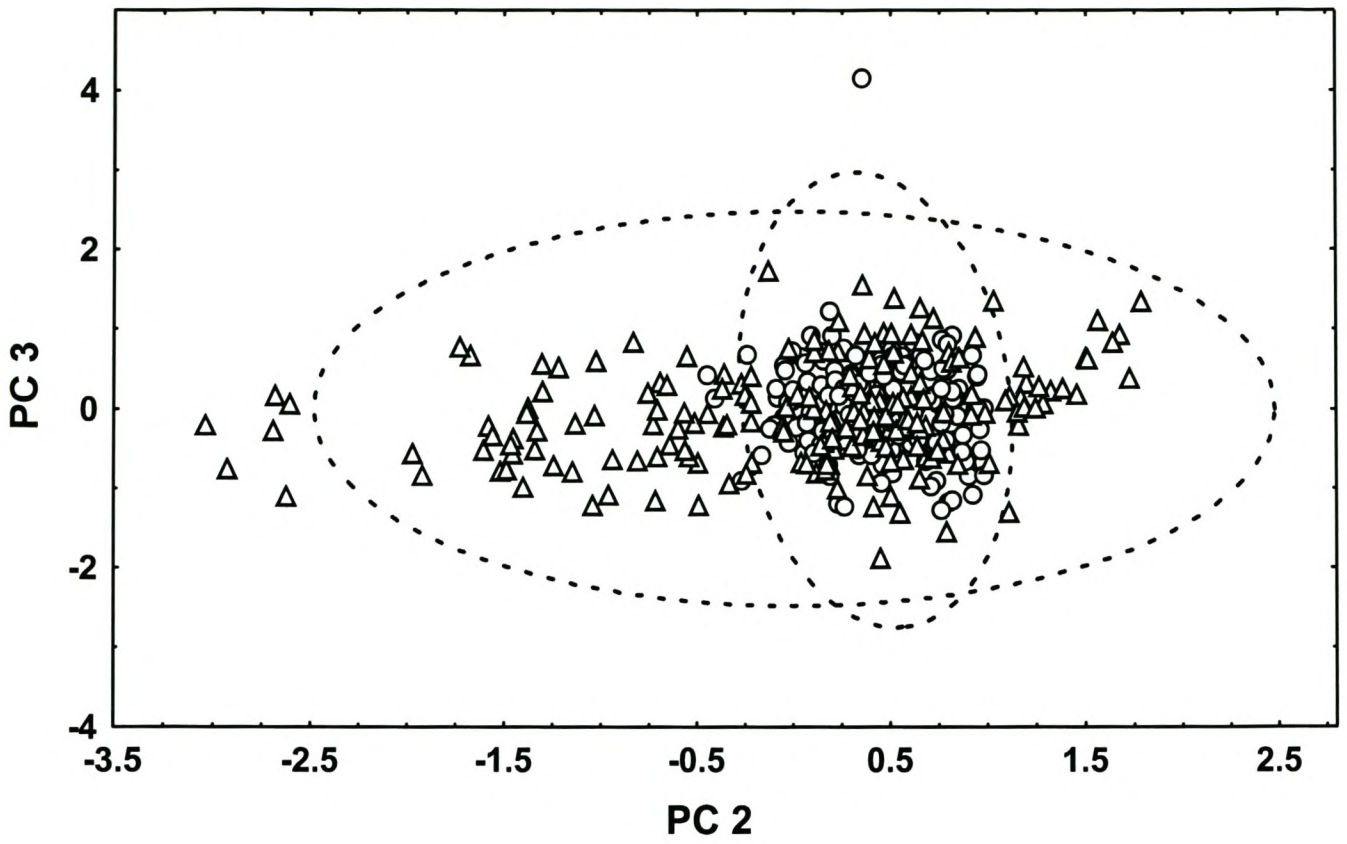


Figure 4. 3. Plot of the second (PC 2) and third (PC 3) principal components for the two taxa, (Δ) *Potamonautes warreni* and (○) *P. unispinus*.

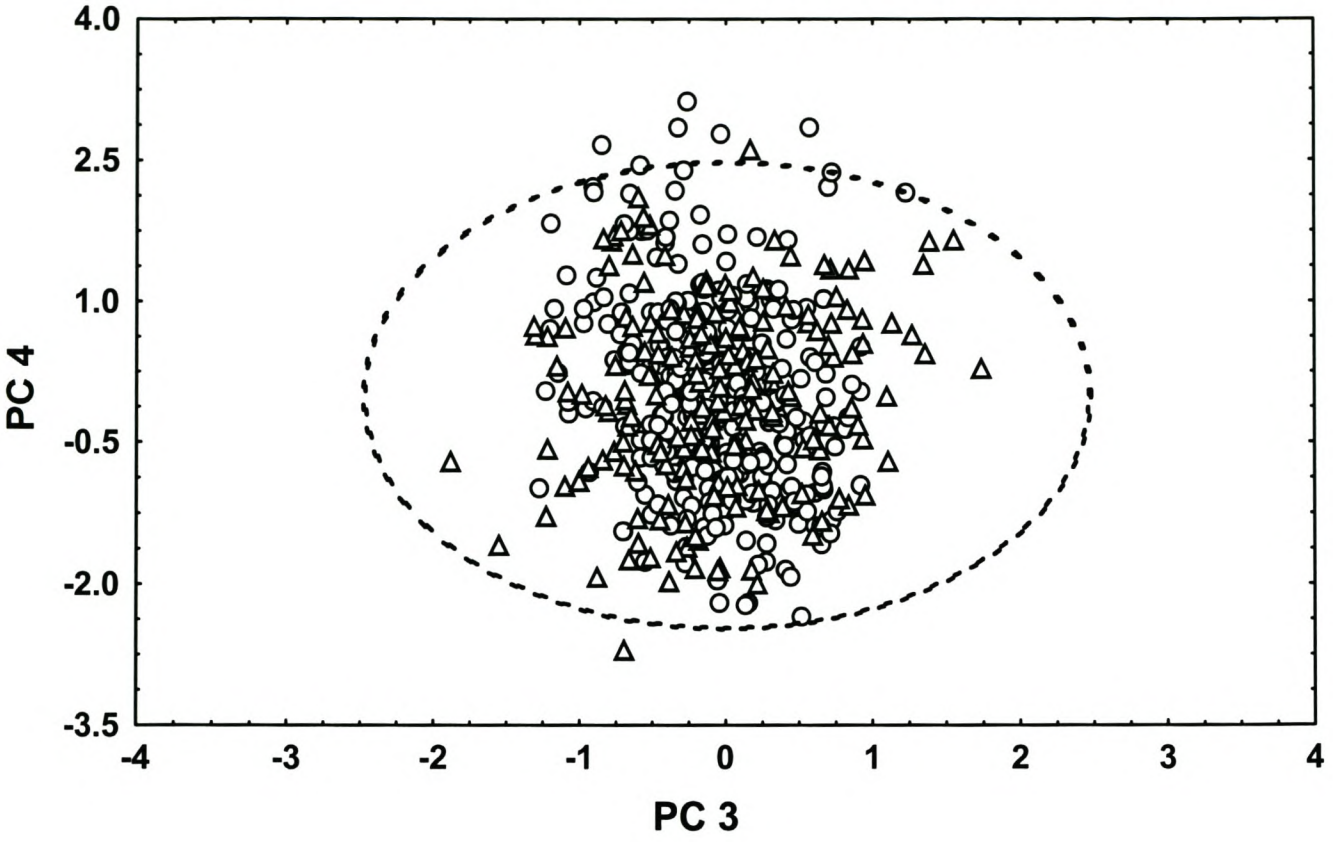


Figure 4. 4. Plot of the third (PC 3) and fourth (PC 4) principal components for the two taxa, (Δ) *P. warreni* and (○) *P. unispinus*.

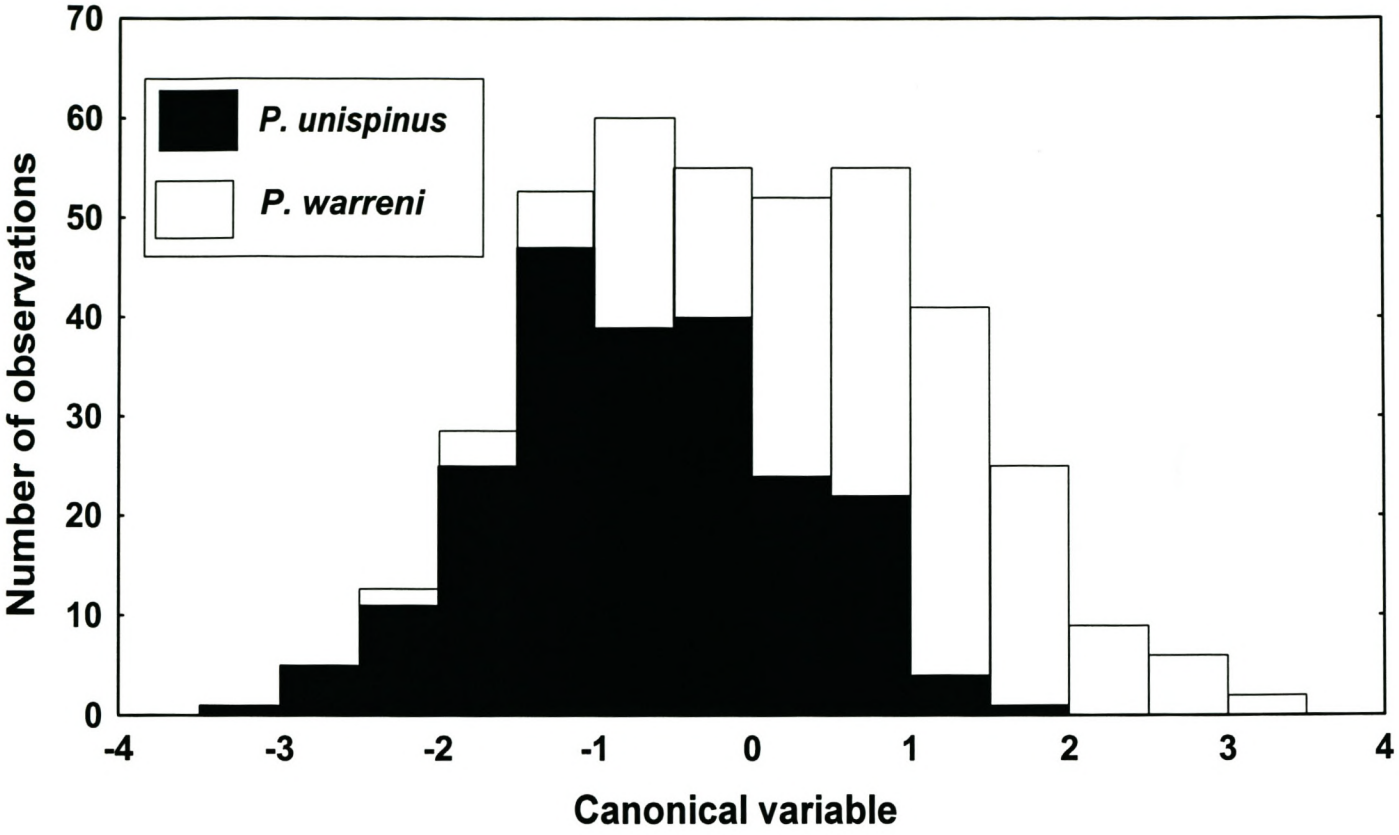


Figure 4. 5. Histogram of scores for *Potamonautes warreni* and *P. unispinus* along the canonical variable calculated from the discriminant function analyses.

A classification function (a linear combination of variables that best discriminates between the groups) was calculated, and this classification for *Potamonautes warreni* was Y = -0.2051 (CL) -1.6544 (CWW) - 0.3059 (CWP) + 0.3417 (PFCD) and for *P. unispinus* was Y = - 0.0813 (CL) - 1.8958 (CWW) - 0.2453 (CWP) + 0.4074 (PFCD). When specimens in the analyses were reassigned to a group with the highest posterior probability using this classification, 67.1 % *P. warreni* specimens (147 of 219 individuals) were correctly reclassified, and 78.2 % *P. unispinus* specimens (223 of 285 individuals) were correctly reassigned to their group. The 11 % difference in accuracy might be accounted for by the 285 individuals included in the *P. unispinus* sample, compared with 219 *P. warreni* individuals sampled. These results indicate a moderate degree of discrimination between the two groups.

The analyses of covariance with carapace length (CL) as an independent variable for the carapace measurements showed a moderate degree of differentiation. The results with CL as an independent variable were not significant between the two taxa for the following measurements: CH ($F = 1.267$; $P = 0.2606$); CWA ($F = 2.543$; $P = 0.1113$); ED ($F = 0.4021$; $P = 0.5262$) and PFCD ($F = 1.5305$; $P = 0.2165$). However, when measurements of the two taxa were statistically compared against CL, the two taxa were significantly different in CWP ($F = 4.456$; $P = 0.0351$) and CWW ($F = 11.740$; $P < 0.001$). Individuals of *P. unispinus* were proportionally broader in the posterior carapace width (CWP) compared to *P. warreni* of similar size (fig. 4. 6a). *Potamonautes warreni* was proportionally broader in the carapace widest width (CWW) when compared to *P. unispinus* individuals of a similar size (fig. 4. 6b).

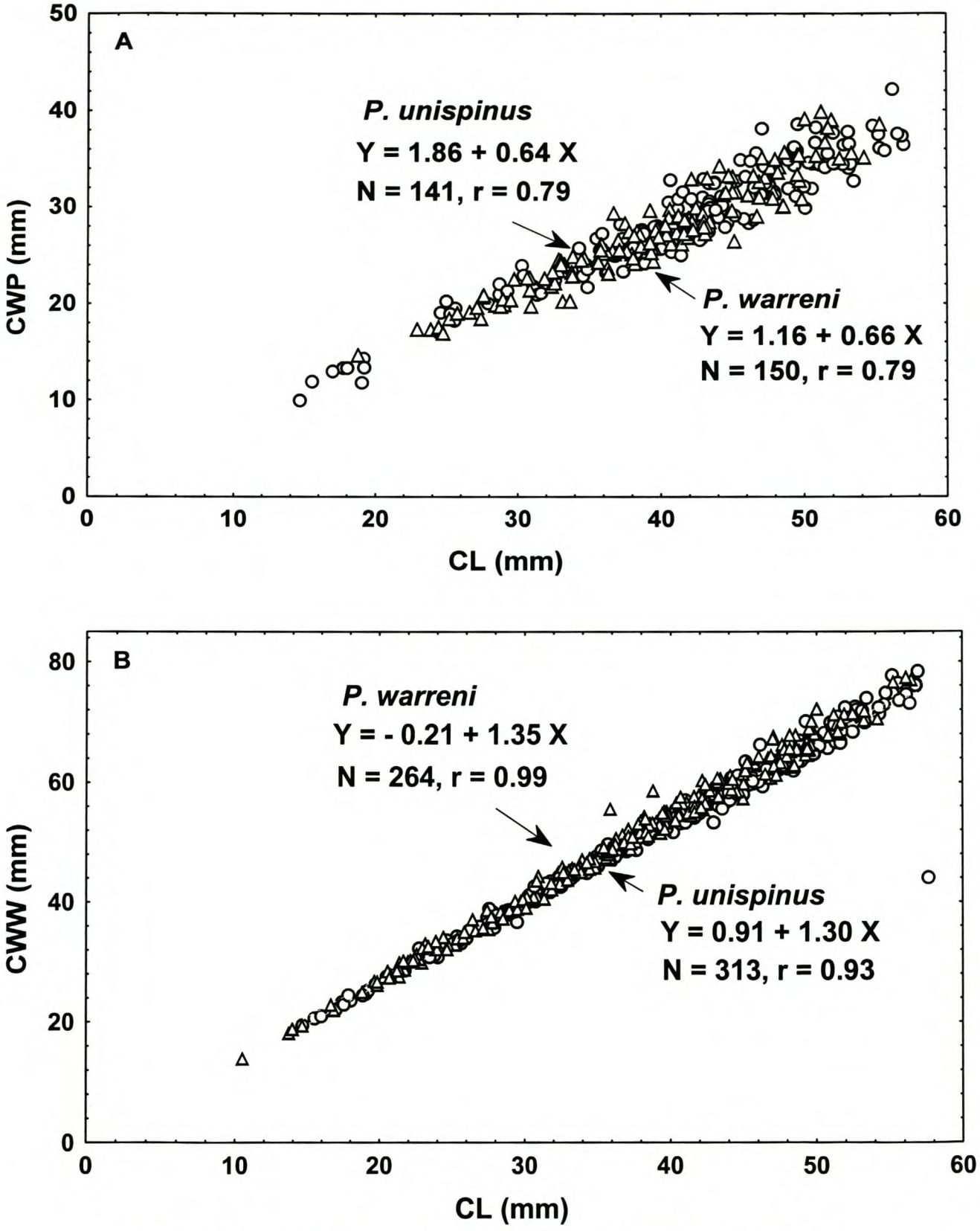


Figure 4. 6. Plot of the carapace length (CL) against carapace widest width posterior (CWP) (A); plot of carapace length (CL) against carapace widest width (CWW) for *Potamonautes warreni* (Δ) and P. unispinus (○) (B).

When P2PL was used as an independent variable and compared to P2PW for the two species, statistically significant results were obtained ($F = 11.00$; $P < 0.001$), indicating that the two species could also be distinguished on the basis of the relative width of their limbs. *Potamonautes unispinus* has proportionally broader limbs when compared to *P. warreni* (fig. 4. 6c). Non significant results were obtained between the two taxa when P2ML was used as an independent for P2MW ($F = 0.442$; $P > 0.05$), when P5ML was used an independent variable for P5MW ($F = 0.3948$; $P > 0.05$) or when P5PL was compared with P5PW ($F = 0.0024$; $P > 0.05$).

Qualitative differences

Based on the differences in the carapace morphology and gonopod 1 shape, it was evident that two distinct morphotypes were present (fig. 4. 7). The carapace structure of *P. unispinus* is conserved and a single well-defined tooth on the anterolateral margins of the carapace always defined this taxon. However, *P. warreni* specimens were highly variable in carapace dentition. Typically, populations had a mean number of 5 teeth on either side of the anterolateral margins of the carapace (fig. 4. 8) but in some instances populations with a single epibranchial tooth were present. Of the *P. warreni* populations examined, 14 % (3 of the 21) of the populations possessed the single tooth form. The mean number of teeth on the left (L) and right (R) hand margin of the carapace for the three population groups were 5. 84 (L) and 6.15 (R) for group one; 6.31 (L) and 6.41 (R) for group two; and 4.45 (L) and 4.54 (R) for group three. No significant difference was observed between the mean number of teeth for the three population groups ($P = 0.80$).

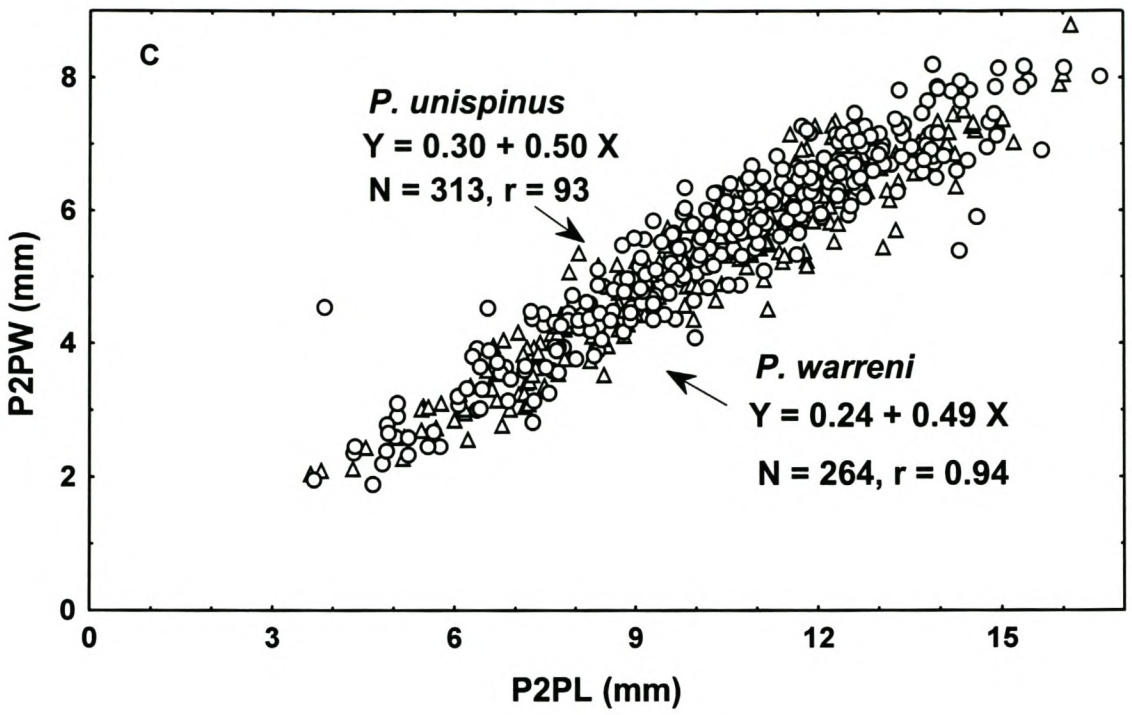


Figure 4. 6. Plot of pereopod 2, propodus length (P2PL) against propodus width (P2PW) for *Potamonautes warreni* (Δ) and *P. unispinus* (O) (C). Scale line = 10 mm.

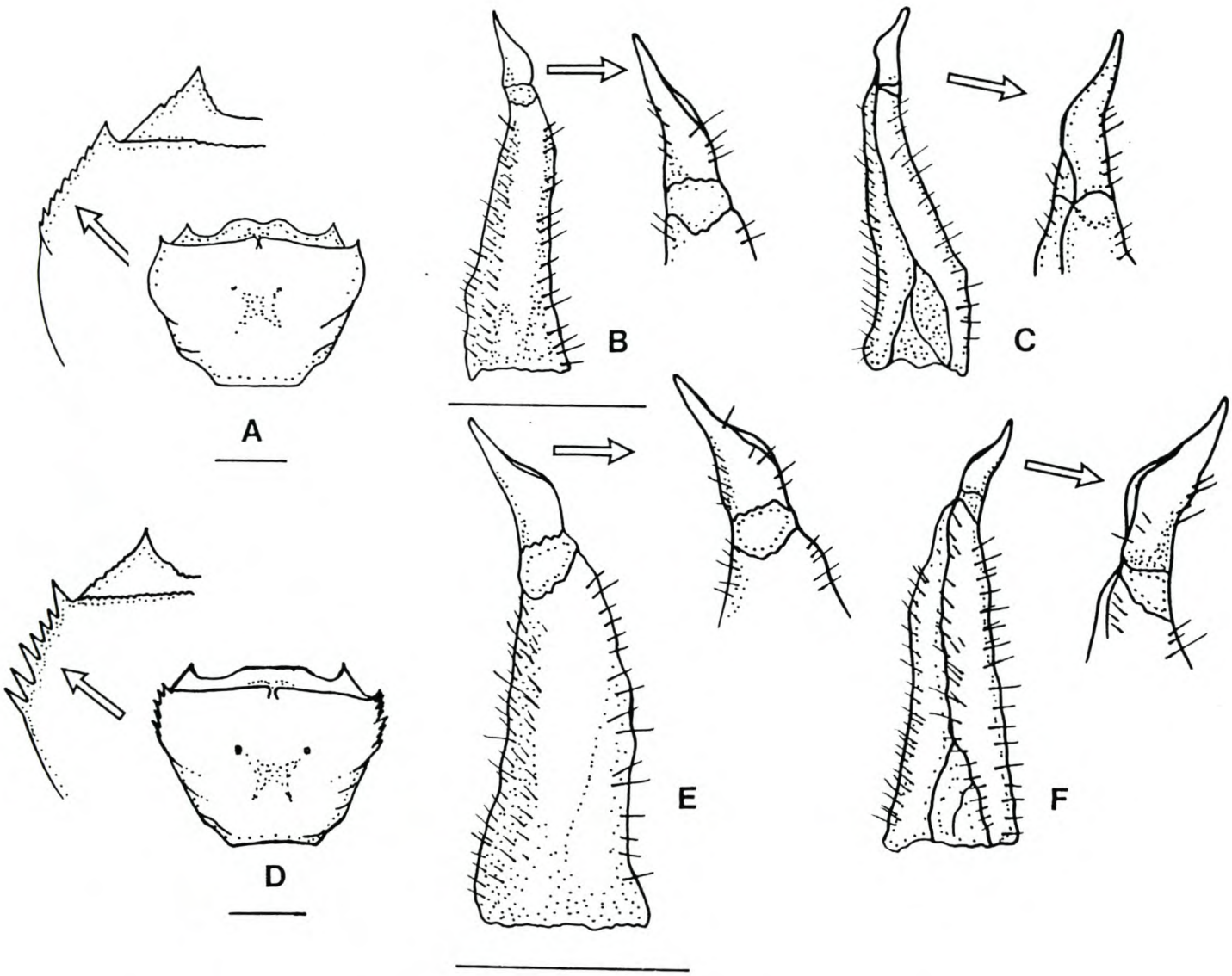


Figure 4. 7. A-E, *Potamonautes unispinus* (Stewart and Cook, 1998), A, carapace outline; B, left gonopod 1, anterior (dorsal) view; C, left gonopod, posterior (ventral) view. *P. warreni* (Calman, 1918), D, carapace outline; E, left gonopod 1 anterior (dorsal) view; F, left gonopod, posterior (ventral) view. Scale bars for the chelipeds and carapace= 10 mm. The scale bar for the remaing features are = 1mm.

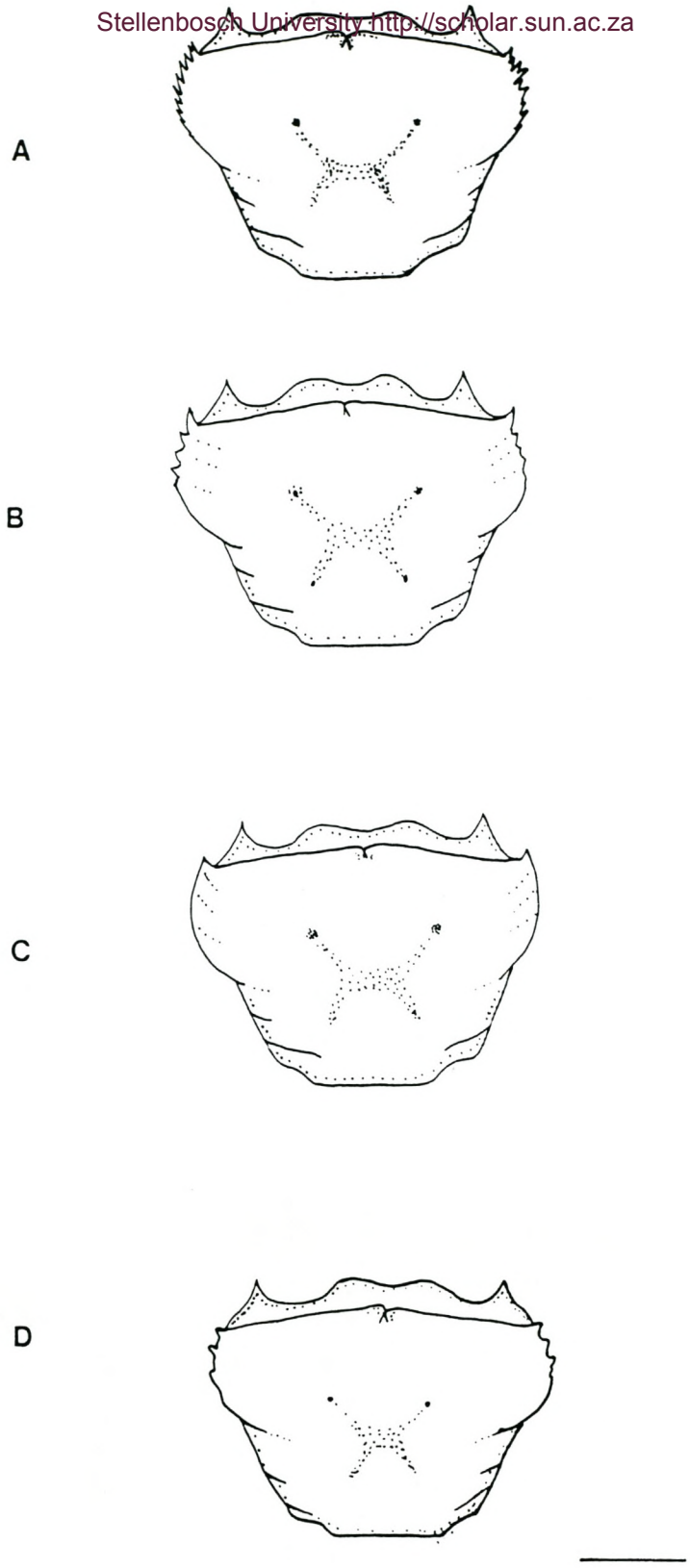


Figure 4. 8. Carapace outline demonstrating the variation in dentition patterns among specimens of *P. warreni* from the Lenasea. Scale bar = 15mm.

The number of teeth on the carapace margin was similar for males and females (P = 0.928). Similarly, there was no significant difference between the number of teeth on the left and right margins between sexes (P = 0.119). The number of teeth on the left and right margins was statistically not significant among females regardless of the size class (P = 0.762), but for males there was a significant difference in the number of teeth between size classes (P = 0.043) (fig. 4. 9). The variation in dentition patterns observed amongst *P. warreni* populations does not exhibit any distinct geographic patterns. Populations of *P. warreni* that exhibit the single toothed form appear to be confined to small streams. *Potamonautes unispinus* is clearly more widespread than *P. warreni* and commonly occurs in the Limpopo and Sabie River systems including its tributaries, and has also been collected further southwards in streams that form part of the Orange River system, while *P. warreni* is restricted to the Orange River system and its major tributaries (fig. 4. 10).

Electrophoretic analyses

Of the 17 loci scored, nine were monomorphic *(Ark-1, Ldh-1, Sdh-1, Gap-1, Mdh-1, Idh-2, Me-1, Mpi-1* and *Pgm-2*) and eight were polymorphic, the allele frequencies for these loci are presented in appendix 2. The number of alleles ranged from two in *Lt-1, Lt-2, Pgd-1, Mdh*-2, and *Idh-1*, three in *Gl*, and four in *Gpi -1* to six in *Pgm -1*. No single locus was polymorphic in all populations. The *Lt-2* locus showed a fixed allelic difference between the two taxa. Fifteen (35, 7 %) of the 42 cases of polymorphism were out of Hardy-Weinberg equilibrium ($P < 0.05$), due to a deficit of heterozygotes carrying the rare alleles. The following populations were out of Hardy-Weinberg equilibrium for the locus identified: *Pgm -1* (χ^2 = 17. 06) in Upington;

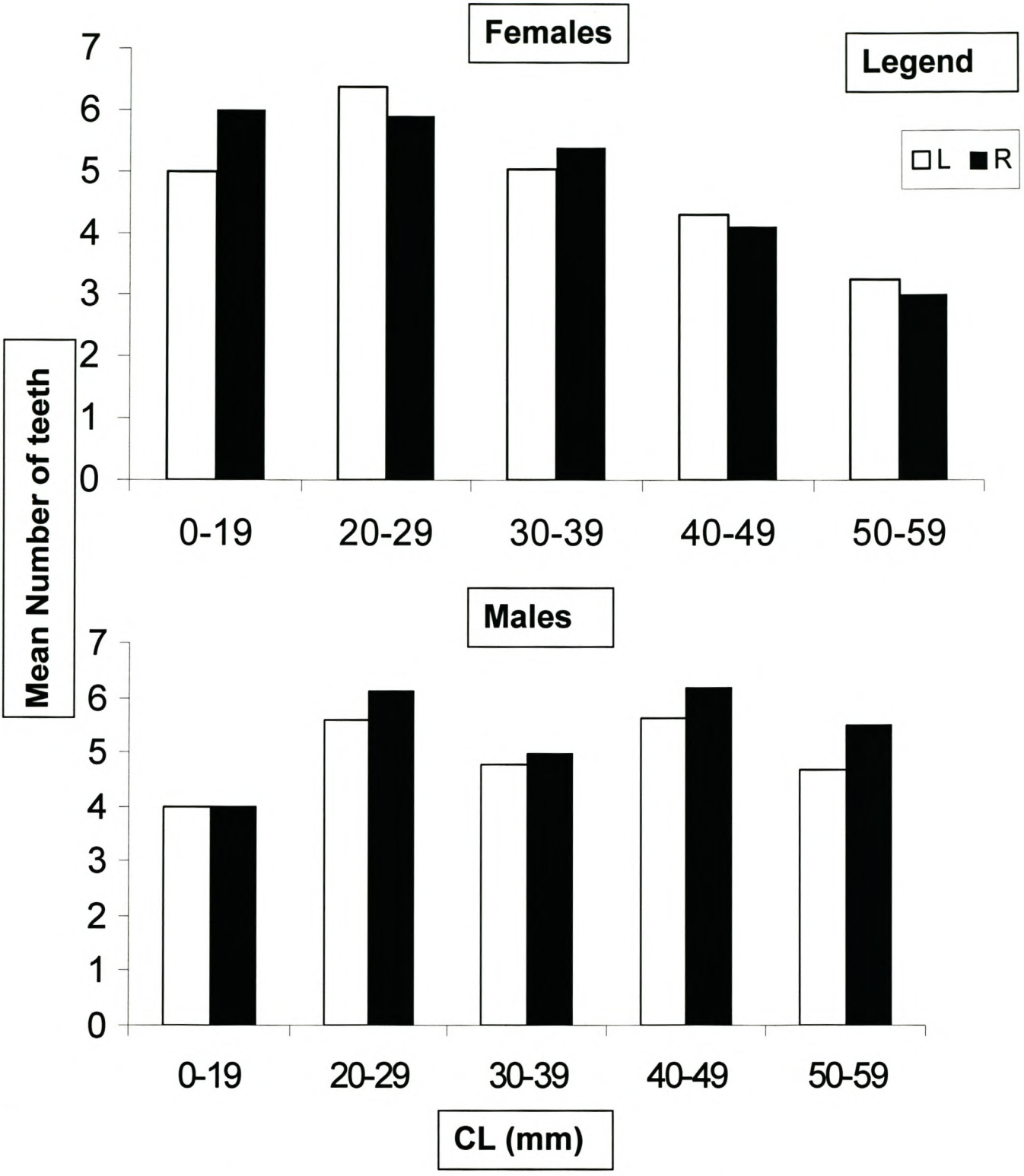


Figure 4. 9. Histogram showing the distribution of the mean number of teeth for each of the size classes between female and male specimens of *P. warreni*. L = left margin, R = right margin

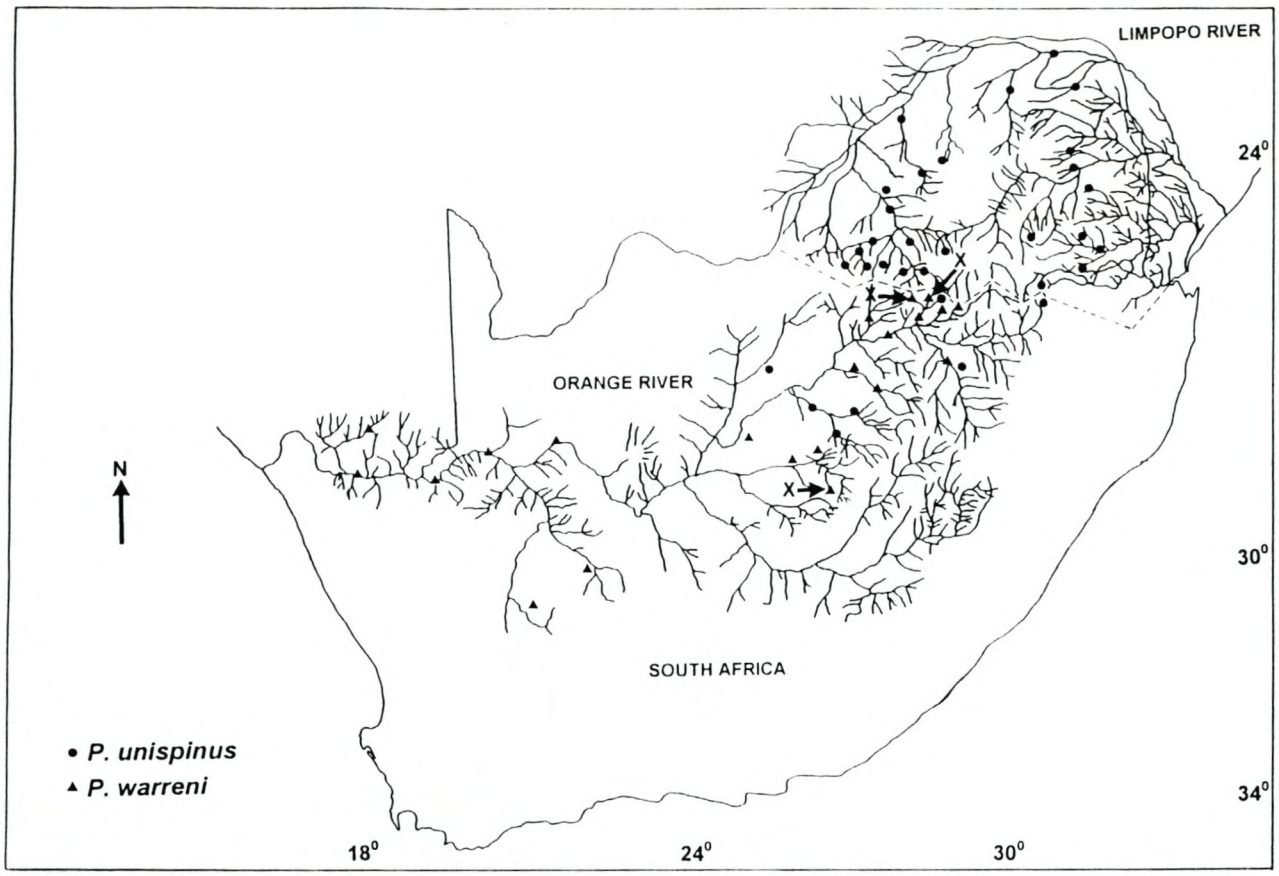


Figure 4. 10. Map showing the distribution of typical *P. warreni* (▲) and *P. unispinus* (●) populations. *P. warreni* populations that are variable in carapace morphology (as in fig. 4. 8) are marked with X.

Pgm -1 (χ^2 = 25.04) in Lenasea; *Pgm -1* (χ^2 = 12.94) and GL-1 (χ^2 = 37.02) in Vaal B; *Pgm -1* (χ^2 = 9.96) in Mbwedi; *Pgm -1* (χ^2 =15.38) in Hex River; *Pgm -1* (χ^2 = 10.54) in the Suikerbos Rand Reserve; *Lt-1* (χ^2 = 19.40) and *Pgm -1* (χ^2 = 9.51) in Elands River; *Pgm -1* (χ^2 = 9.75) in Sterkstroom; *Pgm -1* (χ^2 = 13.03) in Vals River; *Pgm -1* (χ^2 = 23.04) in the Suikerbos Rand River; *Lt -1* (χ^2 = 18.08) in the Crocodile River; *Pgm -1* (χ^2 = 10.15) in the Zout River and *Pgm -1* (χ^2 = 14.51) in the Pienaars River. The mean observed heterozygosity (H_o) was low and ranged from 0.000 to 0.036, and from 0.000 to 0.063 in *P. warreni* and *P. unispinus* respectively (table 4. 3). Levels of heterozygosity were slightly higher in *P. unispinus*. The percentage polymorphism ranged from 0.00 to 17. 6 %. The mean genetic identity values (*I*) at which the two taxa separated was *I* = 0.927 while the intraspecific genetic differentiation varied between 0.976 and 1.00 in both *P. warreni* and *P. unispinus*. The dendrogram clearly separated populations of the two species into two groups (fig. 4. 11). The pairwise $F_{(ST)}$ among *P. unispinus* populations ranged from 0.366 to 0.025, with a mean of all the loci at 0.328. The mean $F_{(IS)}$ ranged from -0.027 to 0.874 with a mean at 0.204 while the $F_{(IT)}$ ranged from -0.001 to 0.887 with a mean at 0.465. The pairwise $F_{(ST)}$ among *P. warreni* populations ranged from 0.026 to 0.459, with a mean of all the loci at 0.417. The mean $F_{(IS)}$ ranged from -0.033 to 0.426 with a mean at 0.344, while the $F_{(IT)}$ ranged from -0.007 to 0.690 with a mean at 0.617. *Potamonautes warreni* appears to be more genetically substructured when compared to *P. unispinus*.

Table 4. 3. The mean number of alleles per locus, the percentage of the loci that were polymorphic and the mean heterozygosity among the populations. Populations of *P. warreni* marked with asterisk, unmarked populations are *P. unispinus*.

Population	Mean sample size	Mean no. of alleles per locus	Percentage polymorphic	Mean heterozygosity
Upington*	19.4	1.1	11.8	0.009
Lenasea*	28.2	1.1	5.9	0.000
Olifants vlei*	30.1	1.1	5.9	0.000
Vaal B	18.5	1.2	17.6	0.034
Shingwedzi	6.6	1.1	5.9	0.034
Mbwedi	14.4	1.4	35.3	0.029
Mutamba	15.3	1.1	11.8	0.063
Edenvale	19.4	1.3	11.8	0.029
Blesbokspruit*	12.7	1.1	5.9	0.022
Vereeniging*	19.7	1.1	5.9	0.006
Hex River	11.9	1.1	5.9	0.027
Sand River	11.6	1.1	5.9	0.012
Suikerbosrand Nature Resurve	16.9	1.1	5.9	0.033
Elands River	15.9	1.2	11.8	0.031
Bothaville*	16.0	1.1	5.9	0.007
Sterkstroom	24.3	1.2	11.8	0.011
Haterbeespoort dam	12.9	1.2	11.8	0.046
Renosterspruit*	12.0	1.0	0.0	0.000

Table 4. 3. continues

Population	Mean sample size	Mean no. of alleles per locus	Percentage polymorphic	Mean heterozygosity
Vredespruit	8.0	1.0	0.0	0.000
Frankfurt	12.0	1.1	5.9	0.005
Suikerbosrand River*	12.0	1.1	5.9	0.000
Crocodile River	13.8	1.2	17.6	0.015
Klip River*	15.5	1.2	17.6	0.036
Zout River	12.8	1.1	5.9	0.000
Rustenburg Nature Reserve	13.7	1.1	5.9	0.013
Villiers*	14.8	1.1	11.8	0.028
Pienaars River	19.0	1.2	17.6	0.037
W.P. Gamepark	16.0	1.1	5.9	0.004

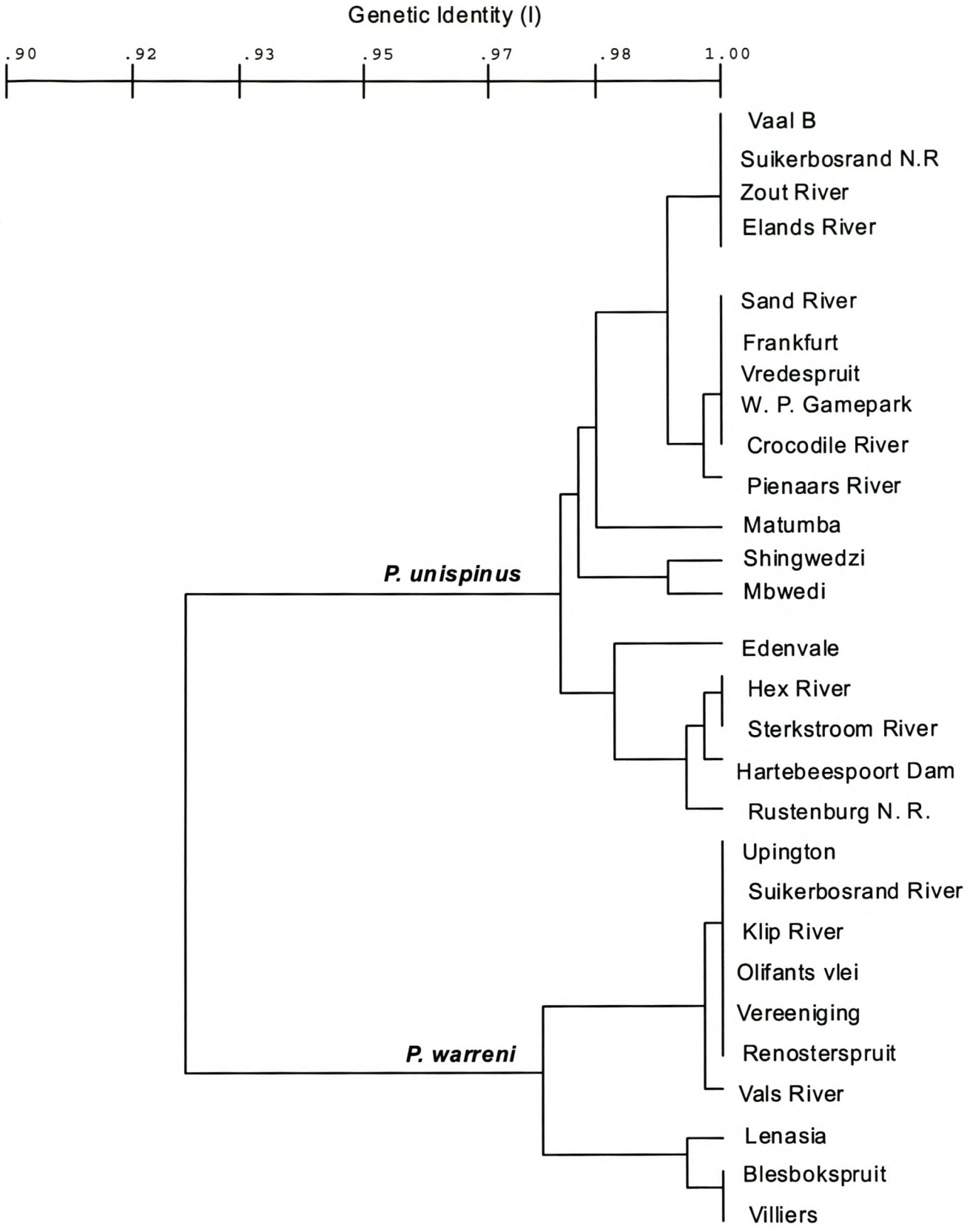


Figure 4. 11. Dendrogram generated from a matrix of Nei's (1978) genetic identities and the UPGMA algorithm based on the 17 loci.

Discussion

In the present study the morphometric, morphological and genetic data support the taxonomic separation of *Potamonautes warreni* and *P. unispinus*. The multivariate morphometric analyses indicated shape differences between the taxa, while the morphological features (carapace dentition and gonopod 1) could consistently distinguish the two taxa. A genetic identity (*I*) value of 0.92 was reported for the two taxa. Although this genetic *I* value is high, it is comparable with some *I* values obtained for other interspecific comparison of river crab populations. For example, *P. sidneyi* and an undescribed species from KwaZulu-Natal, South Africa separated at a *I* value of 0.82 (Gouws, 1999), while an *I* value of 0.88 was reported between *P. perlatus* and *P. granularis* (Daniels *et al.*, 1999a), and *P. perlatus* and *P. sidneyi* separated at an *I* value of 0.83 (Gouws, 1999). Generally, however, genetic *I* values for interspecific potamonautid crab populations are considerably lower and include an *I* value of 0.66 between *P. brincki* and *P. perlatus*, (Stewart, 1997a), 0.68 between *P. parvispina* and *P.perlatus*, (Stewart, 1997b) and an *I* value of 0.66 between *P. sidneyi* and *P. unispinus* (Stewart and Cook, 1998). What is interesting about the latter group of species pairs is that they are morphologically distinct. In contrast, taxa that are morphologically very similar in appearance such as *P. warreni* and *P. unispinus; P. granularis*, *P. perlatus* and *P. sidneyi* appear to exhibit higher levels of genetic similarity.

These results compare well with other allozyme studies among marine decapod crustaceans. For example, Tam and Chu (1993) reported a genetic *I* value of 0.86 separating the shrimps *Penaeus merguiensis* and *P. pencillatus*, and an *I* value of 0.92

between *P. semisulcatus* and *Metapenaeus affinis*, while Chu, Lee and Tsoi (1990) reported a genetic *I* value of 0.88 between *Metanephrops formosanus* and *M. japonicus*. According to Thorpe (1982) and Thorpe and Sole-Cava (1994) genetic *I* values for congeneric invertebrate taxa are generally < 0.85, while conspecific groups have *I* values > 0.85. However, these authors warn that *I* values should not be used as absolute values, but rather as a guideline to discern species from populations. Genetic identity values for 28 congeneric decapods ranged from (*I*) 0.36 to 0.92 (Hedgecock, *et al.,* 1982) and indicated that I value may be widely variable.

If the biological species concept is applied here, the two taxa - although genetically closely related - are two distinct species. Although the two taxa were not found in sympatry in the present study, populations of *P. warreni* and *P. unispinus* have been found in close geographic proximity that would allow for gene exchange. For example, populations of both taxa were found in continuous tributaries of the Klip River system in the Gauteng Province. The lack of geographic barriers within this system would allow for gene exchange to occur; however this was not evident. The presence of a diagnostic allelic difference at *Lt-2* for both taxa, and the absence of hybrids, indicate a lack of recent gene flow, and thus genetic isolation. The two taxa thus fit the biological species concept (Bock, 1992). Bock (1992) argues that species may not always have all the attributes (genetic, reproductive and ecological isolation) in place to fit the biological species concept and he suggests that ecological isolation may play a significant part in preventing genetic homogeneity between taxa. The phylogenetic species concept defines a species as "the smallest diagnostic cluster of individual organisms within which there is parental patterns of ancestry and descent"

(Cracraft, 1989, 1997). The difference in the gonopods and carapace dentition between *P. warreni* and *P. unispinus* may represent such heritable traits.

The high genetic similarity between the two species is likely to be attributed to a recent speciation event if we apply the two molecular clocks calibrated by Nei and Roychoundrung (1974) and Yang *et al.*, (1974). If our data ($D < 0.08$) is extrapolated to these values the time of speciation is likely to be recent (Pleistocene / Holocene). Conspecific river crab populations are generally characterised by genetic invariability over considerable geographic distance and *I* values > 0.97 have been recorded (Daniels *et al.,* 1998a, 1999b). Similar results have also been reported in marine decapod crustaceans (Nelson and Hedgecock, 1980; Seeb *et al.,* 1990) and seem to be a common occurrence among decapod crustaceans. The low mean heterozygosity observed in the present study compares favourably with those reported in other freshwater crabs (Aotsuka *et al.*, 1995; Daniels *et al.*, 1998a, 1999a,b; Gouws, 1999) yet these results were generally considerable lower than the mean value of 0.048 obtained for decapod crustaceans (Hedgecock *et al.*, 1982) and studies on marine decapods (Stevens, 1990; Felder and Staton, 1994; Passamonti *et al.*, 1993; Staton and Felder, 1995; Creasey *et al.*, 1998; Maltagiati *et al.*, 1998). However, low heterozygosity appears to be a common feature among freshwater crabs. The $F_{(ST)}$ indicates that *P. warreni* is genetically more structured than *P. unispinus*, despite the fact that *P. unispinus* is distributed in three separate river systems, while *P. warreni* is confined to a single river system.

The lack of considerable genetic structure between populations is not surprising as

freshwater crabs are well known for there amphibious nature and have been observed to migrate short distances over land during favourable conditions, such as flooding (Barberesi *et al.*, 1997). *Potamonautes warreni* is a species thought to be endemic to the Orange River system and its tributaries where there are limited barriers to gene flow. On the other hand, *P. unispinus* was characterised by considerable heterogeneity at *Pgm -1*, with a number of populations possessing rare alleles. This may be attributed to limited gene exchange, probably the effect of habitat segregation between populations, as *P. unispinus* is distributed over three catchments. The genetic variation at *Pgm -1* and *Gpi -1* may be the result of spatially and temporally chaotic distribution of allele frequencies, in which local variation seemingly exceeds long-distance variation, a similar pattern having been reported for a number of invertebrates (Benzie and Stoddart, 1992; Campton *et al.,* 1992; McMillen-Jackson *et al.,* 1994). The low overall within-population genetic variation of both taxa is likely to reflect the genetic legacy of the original founder population, while recent and ongoing evolutionary processes (drift and selection, gene flow) are likely to have contributed to the observed within population divergence. These results suggests that animals such as river crabs, that exhibit direct development (and as a rule lack planktonic larvae), are not necessarily highly structured genetic units, due to the high dispersal capacity of adult crabs. Alternatively, the allozyme markers may be too conservative to detect intraspecific genetic variation among river crabs. Indeed, DNA sequencing of a rapidly evolving mitochondrial gene has revealed historic patterns of genetic differentiation on a micro-and macro-geographic scale for a wide range of crustaceans (Bucklin *et al.*, 1995; 1997; Cuesta and Schubart, 1998) and may thus be informative in detecting population differentiation among river crabs. Specimens

resembling *P. unispinus* have been recorded from river systems in Zambia (Stewart, pers. comm.), indicating that this species possibly has a wider tropical distribution and has probably migrated into South African river systems through river capture, and under more favourable wetter conditions during the Quanternary (Wild, 1968). *P. warreni* is likely to be a tropical species confined to the Orange River system. Similar distribution patterns have been recorded for the freshwater fishes of South Africa (Skelton, 1994).

Morphologically, the two species are distinct. *Potamonautes unispinus* is characterised by a single well defined tooth on the anterolateral margins of the carapace, while *P. warreni* is highly variable in dentition patterns and is "typically" characterised by 5 -10 well defined teeth to individuals with as single epibranchial tooth. The variation in dentition patterns in *P. warreni* populations possesses no genetic bases (as suggested by allozymes) and is likely to be the product of heterogeneous environmental selection pressures (ecogenesis) between the localities. In the present study, no differences in the dentition pattern between sexes were observed, indicating that dentition may not play a role in mate recognition. The variation in dentition patterns between sites may be attributed to the result of abiotic factors (water temperature, changes in the waters chemical composition or changes in the flow regime) and biological factors (food availability and predation). These factors are well known to cause morphometric variation among sites between crustacean populations (Bas and Sarda, 1998) and other aquatic invertebrates (Katoh and Foltz, 1994). Although these parameters were not examined in the present study, it is likely that certain of these factors may have led to the current variation in dentition patterns

between *P. warreni* populations. Overton *et al.,* (1997) remarked that meristic data such as the presence of spines might be a weak character in identifying crustacean species. For example, dentition in the mud crab, *Scylla serrata* may be worn down as the animal increases in size, thus making it difficult to count the number of teeth with accuracy. These authors noted that spines were often broken and subject to developmental variation.

Despite the fact that morphological variation between river crabs is well documented (Alcock, 1909a, 1910; Barnard, 1935, 1950; Bott, 1955), few large-scale intraspecific morphological studies have been conducted on river crabs, to assess the value of such morphological characters. Bott (1955) and other authors reported considerable variability in the carapace dentition patterns amongst the African *Potamonautes*. For example teeth can be either present or absent, while juveniles and adults can differ in the number of teeth (*P. langi,* Rathbun, 1921). Variability in diagnostic dentition patterns is not unique to the Potamonautidae, and has also been reported in the East Africa freshwater crab family, Plathyhelphusidae (Cumberlidge, 1999b), as well as amongst genera of the South American freshwater crab family, Trichodactylidae (Rodriguez, 1992a). For example, Rodriguez (1992a) reported that in *Microtrichodactylus panoplus* (von Martens, 1869) that the dentition pattern vary between four to five spines, while in *Dilocarcinus dentatus* (Randall, 1839), the teeth varies between eight to ten, and becomes blunt with maturity and in *Forsteria venezuelensis* (Rathbun, 1906), the young specimens possesses three teeth on the anterio-lateral margins, that becomes obsolete in older males. Evident from the earlier taxonomic studies on river crabs is that the terminology employed to describe species

and distinguish species was often applied with inconsistency. This problem has remained till the present day, and has contributed to the current chaotic state of river crab systematics. As a direct consequence the systematic affinities of species the genus *Potamonautes* is doubtful and in need of revision.

In South Africa, no other toothed river crab species is known to exhibit such considerable morphological variation as that recorded in *P. warreni*. For example *P. parvispina* (Stewart, 1997a) and *P. unispinus* may consistently be distinguished from other river crabs by a single tooth on the anterolateral margins of the carapace, while *P. dentatus* (Stewart *et al.*, 1995) is always characterised by several prominent teeth on the anterolateral margins of the carapace. Bott (1955) recorded at least 16 toothed river crab species in *Potamonautes*. The systematics of many of these species needs to be examined to determine if they constitute species or morphological varieties of widespread taxa. The use of dentition as a species discriminatory character among river crabs appears to be reliable in discerning species, but it may in certain instances be a variable character. Where dentition patterns are highly variable, such as in *P. warreni* this character should be evaluated and used in combination with other morphological data (gonopod and mandibular structures) and corroborated with genetic data to determine species boundaries.

Chapter 5

Allometric growth, handedness, and morphological variation in *Potamonautes warreni* (Calman, 1918) (Decapoda, Brachyura, Potamonautidae) with a re-description of the species

Abstract

Some morphological relationships are investigated amongst three populations of *Potamonautes warreni* (Calman, 1918) and the species is redescribed. Differences between sexes as well as the growth of the carapace, chelipeds, and handedness are quantified. Sex ratios did not differ significantly from 1:1 among sites, and no differences in the carapace variables were evident between sexes. Both sexes were heterochelic, with the right chela usually being larger in males than in females. Sexual dimorphism is evident for the width of the abdominal somites. The functional significance of an enlarged right chela, and that of patterns of allometric growth are discussed. In addition, the structure of pleopod 1, mandibular palp, chelipeds, and the third maxilliped are described and illustrated. The distribution of *P. warreni* is re-examined and the species appears to be restricted to the Orange River System and its major tributaries such as the Vaal River in South Africa and Namibia.

Introduction

Crabs of the family Potamonautidae are common in freshwater systems throughout South Africa (Barnard, 1935, 1950; Bott, 1955). All the freshwater crabs described from this region belong to the genus *Potamonautes,* which is the most speciose of the five genera in this family (Cumberlidge, 1999a). Calman's (1918) original description of *P. warreni* was based on a female collected from Potchefstroom in Transvaal (now, North West Province), South Africa. The original description consisted of only a brief note on the carapace morphology, without any reference to the taxonomically-important gonopods, mandibular palp, maxillipeds, or chelipeds. As a result, the morphological features that distinguish *P. warreni* from other potamonautid river crabs remained unknown, warranting a more comprehensive description. More recently (chapter, 4) noted that the dentition pattern commonly used to distinguish *P. warreni* from other freshwater crabs is highly variable among certain populations. These authors noted that the dentition pattern in this species may range from the presence of a single tooth to 5-10 well-defined teeth on the epibranchial corner of the carapace. Intraspecific patterns of morphological variation in this species have, however, not been investigated, and the degree of morphological variation among populations of *P. warreni* is consequently unknown. Few detailed morphometric studies of freshwater crabs exist. As in other heterochelous crabs, one claw is often enlarged, and generally thought to play a role in food acquisition and crushing. Yet, the functional significance of an enlarged cheliped in freshwater crabs has not been studied. In addition, the changes in overall body morphology have been poorly documented in this group. In the present study, morphological variation among

populations of *P. warreni* is quantified and the diagnostic morphological features of this species are described and illustrated.

Materials and methods

A total of 249 *Potamonautes warreni* specimens of (118 males and 131 females) were collected from three geographical areas of the Orange River system. Group 1 is from the lower section near Upington, group 2 from Bothaville (Vaal River system), and group 3 from Bloemfontein (Vaal River system). The carapace and limbs were measured to the nearest 0.1 mm using digital calipers attached to a portable computer. The following measurements were taken: carapace length along the medial line (CL); carapace width at the widest part (CWW); distance between postfrontal crest and anterior margins of the carapace (PFCD); distance between medial margin of the orbits (ED); distance between the exorbital teeth (CWA); carapace height (depth) (CH); width of the fifth (AW5) and sixth (AW6) abdominal somites: right and left cheliped propodus length (CLPL; CRPL) and width (CLPW; CRPW); length of propodus of pereopod 2 (P2PL); width of propodus of pereopod 2 (P2PW); length of merus of pereopod 2 (P2ML); width of merus of pereopod 2 (P2MW); length of propodus of pereopod 5 (P5PL); width of propodus of pereopod 5 (P5PW); length of merus of pereopod 5 (P5ML) and width of merus of pereopod 5 (P5MW).

The overall sex ratio (S_0) was determined according to the formula modified from Christiansen *et al.*, (1990):

$$S_0 = (M_0 - F_0) / (M_0 + F_0)$$

where M_0 is the number of males in the sample and F_0 the number of females in the

sample. The sex ratio of the crabs was analysed using a two-tailed χ^2 test to determine if there was a significant deviation from an expected 1:1 sex ratio. The effective population size (N_e) was determined according to the equation given in Li and Graur (1991):

$$Ne = 4\ NmNf / Nm+Nf$$

where Nm and Nf are the respective proportions of males and females in a population of N individuals. Student's *t* - test was used to test for significant differences between variables for males and females. Size distribution was plotted for both sexes using 5 mm carapace length intervals. Bivariate scatter plots were drawn for selected variables using the software package STATISTICA (Stat Soft Inc.1996).

The relative growth of crustaceans is generally in accordance with the simple allometry rule, $Y = aX^{b.}$ Traditionally, allometric growth equations describe a power curve that can be linearized by logarithmic transformation to log Y = log a + b log X. Despite arguments against log transformation of variables (Zar, 1968), this transformation has remained widely in use (Finney and Abele, 1981; Abby-Kalio and Warner, 1989; Abell'o *et al.*, 1990). The least square method of regression is also commonly used. However, this method has received criticism (Ricker, 1973, 1975), because it assumes that the independent variable is measured without error. The constant of allometry was determined for a selection of parameters by logarithmic transformation of the data and subsequent log-log regression equations. A Student's *t*-test was used to determine the allometric status of structures against the isometric

standard slope. Analysis of covariance (ANCOVA) was performed on the log-transformed variables and used to test for significant differences between sexes. Scatterplots of the log transformed variables were drawn for females and males.

AWild stereomicroscope was used to examine the right third maxilliped, mandible, mandibular palp, and pleopods (gonopods) 1 and 2. In addition, the carapace outline as well as the dactylus and propodus of the left and right chelipeds of the *P. warreni* holotype, and the gonopods of a male specimen were examined. Abbreviations: BMNH = British Museum (Natural History); SAM = South African Museum, Cape Town, South Africa.

Taxonomy

Potamonautes warreni (Calman, 1918)

Material examined. – One female (holotype) Potchefstroom, Transvaal, collected 1918 by E Warren (BMNH, 1918.3.30.1); one male, Potchefstroom, Transvaal, collected December 1917 by Cawston (BMNH, 1918.5.14.1); one male, one female Vioolsdrift (lower Orange River, South Africa, (SAM A 41143), collected 20 May, 1992 by B. Stewart and L. Hoenson; four males and four females Gifkloof, Orange River, South Africa, collected 21 March, 1994 by R. Palmer and M. Scheepers (SAM A 41150); one female Fish River – near Seehiem, Namibia, collected February, 1969, collector(s) unknown (SAM A 41152); eight males, four females, Modder River, Glen, South Africa, collected date unknown, collected by R Bigalke (SAM A 6358); three males, Zak River, Willinston, South Africa, collected in 1939 by K. Barnard, (SAM A 8345); two females, one male Barberspan, South Africa, collected 6 April,

1928 by Hutchinson (SAM A 6899); one male, Kroonstad, unknown date collected by Brunette (SAM A 10885); one male Rehoboth, Namibia, date and collected unknown (SAM A 10886); two males and two females, Glen, date unknown, collected by R Bigalke (SAM A 6366); three females, one male, Leber River near Gibeon, Namibia, January 1916, collected by R. W.Tucker (SAM A 3953); one female, Warrendon, Vaal River system collected 1939 by Hess, Boonstra and Thorne (SAM A 8393); one female, BarkelyWest, collected date unknown by T Clayson (SAM A 10887).

Type locality. – The holotype was originally described from near Potchefstroom, South Africa, from a tributary that flows into the Vaal River system, a major tributary of the Orange River system.

Distribution. - This species is restricted to the middle reaches of the Orange and Vaal River systems and their major tributaries in South Africa and southern part of Namibia.

Diagnosis. - The anterolateral margin of the carapace usually with a series of eight well-defined teeth; certain specimens possesses a series of 5 to 10 well-defined teeth, while a single epibranchial toothed form has also recently been discovered (fig. 5.1).

Description. – Measurements of the holotype are given in table 5.1. Carapace margins characterized by eight well-defined teeth, branchial region convex, maximum height and width at anterior third (ratio CH/CL = 0.49; CWW/CL = 1.36) (fig 5.1).
Postfrontal crest complete well-developed, granulated and with a groove at midpoint.

Table 5. 1. Measurements (in mm) of the holotype and ranges (for 127 males and 137 females) for *Potamonautes warreni* (Calman, 1918).

Variable	Abbreviation	Holotype	Males	Females
Carapace length	CL	50.12	56.11-13.7	62.52-10.52
Carapace widest width	CWW	68.44	77.33-18.06	82.54-13.89
Carapace height	CH	24.71	28.20-6.57	35.33-4.92
Distance between postfrontal crest and the anterior margins of the carapace	PFCD	8.00	8.06-2.00	9.13-1.64
Distance between orbits	ED	26.36	28.81-7.40	30.93-5.48
Distance between exorbital teeth	CWA	30.79	51.04-15.06	56.30-11.36
Width of six abdominal segment	AW6	28.61	15.15-3.32	16.85-2.44
Major cheliped propodus length	MCPL	47.38	78.29-9.82	56.58-6.65
Major cheliped propodus height	MCPH	19.00	28.72-3.32	21.43-2.54
Pereopod 2, merus length	P2ML	24.18	29.15-6.98	29.64-7.05
Pereopod 2, merus width	P2MW	9.80	9.61-2.92	10.33-3.00

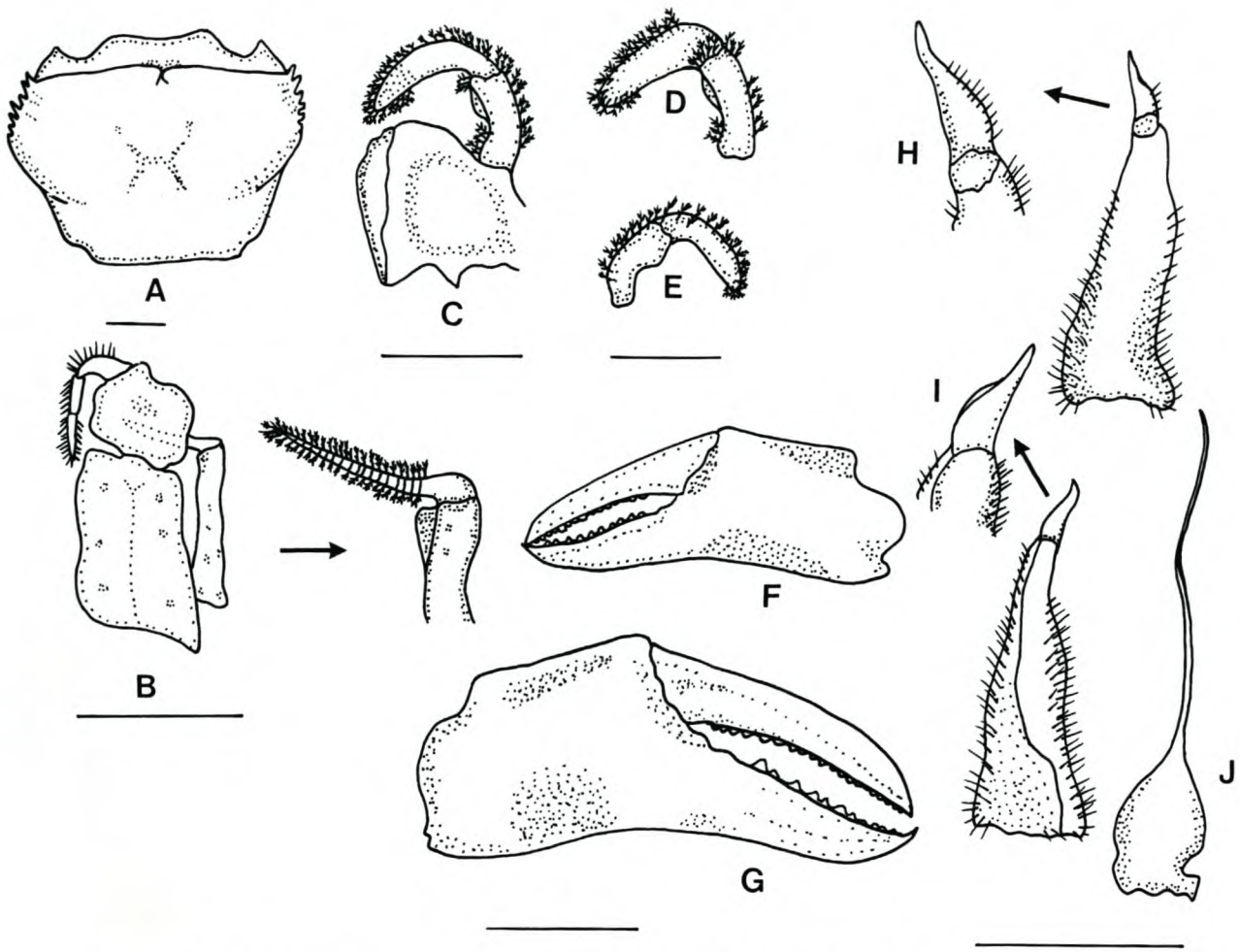


Figure 5. 1. *Potamonautes warreni* (Calman, 1918) A-G female holotype; A, carapace outline; B right third maxilliped, posterior view; C, right mandible and mandibular palp, posterior view; D, right mandibular palp, posterior view; E, right mandibular palp, anterior view; F, left cheliped, dactylus and propodus; G, right cheliped, dactylus and propodus; H to J, from a male specimen from Gifkoof. H, left gonopod 1, anterior view; I, left gonopod 1, posterior view; J, right gonopod 2 anterior view. Scale bars for the chelipeds and carapace= 10 mm. The scale bar for the remaing features are = 1mm.

Anterolateral margins between exorbital and epibranchial tooth finely beaded. Urogastric and cardiac grooves moderately deep and well defined. Sternites 1 and 2 fused, no suture discernible, suture (first sternal groove) between sternites 2 and 3 complete. Second sternal groove between sternites 3 and 4 complete laterally, becoming shallow towards the middle and sloping sharply towards the abdominal area.

Mandibular palp composed of two segments; terminal segment undivided and with a dense tuft of setae on the posterior flange, margins very setose (fig. 5. 1B). The third maxillipeds fill the entire buccal frame, except for a small, oval respiratory opening; flagellum present on exopod, ischium possessing a faint but distinct vertical groove (fig. 5. 1C). Chelipeds unequal, dactylus of right and left chelipeds arched. Dactylus of right cheliped more mildly arched, and 1.08 times length of dactylus of left cheliped. Both dactyli armed with several small to medium sized cutting teeth. Right propodus swollen, 1.12 times longer and 1.29 times wider than propodus of left cheliped, with a series of well-defined teeth. Carpus of both chelipeds with two prominent teeth (fig. 5 .1D). Antero-inferior margins of merus with prominent spine, posterio-inferior margins without a spine. Pereopods broad (P2ML/P2MW = 2.46; P5ML/P5MW = 2.51) with P3 being the longest and P5 the shortest. Dactyli ending in sharp pointed margin with spine-like bristles. Terminal segment of first gonopod short, curving away from the midpoint, wider at the base when viewed posteriorly and ending in a pointed tip. Sub-terminal segment of gonopod 1 tapering distally, with a relatively smooth inner margin, posterior surface with longitudinal groove (fig. 5. 1E). Gonopod 2 terminal segment filamentous and hollow, widest at its base and tapering

off sharply inwards, to form an upright process that supports the terminal segment (fig. 5. 1F).

Variation – The dentition pattern on the anterolateral margin of the carapace may vary from a single tooth in certain specimens to a series of 5-10 well-defined teeth in others. Both sexes exhibit heterochely. Pubertal moult occurring approximately in size classes with CL = 25–33 mm.

Colouration - Carapace and limbs chocolate brown when alive, fading to light brown when preserved in 70 % ethanol.

Remarks - Apart from *Potamonautes warreni*, six of the potamonautid river crab species described in South Africa thus far have epibranchial teeth on the anterolateral margins of the carapace. In *P. dentatus* (Stewart *et al.*, 1995) the anterolateral margin posterior to the epibranchial tooth contain 4 to 5 small but distinct, pointed teeth and the postfrontal crest contains an epibranchial sinus posterior to the exorbital tooth. The terminal segment of gonopod 1 differs between *P. dentatus* and *P. warreni*. These two species are geographically quite distinct, as *P. dentatus* is a KwaZulu Natal endemic. The remaining toothed river crabs have a single epibranchial tooth. These include *P. bayonianus* (Brito Capello, 1864), *P. dubius* (Brito Capello, 1873), *P. calcaratus* (Gordon, 1929), *P. parvispina* (Stewart, 1997a) and *P.unispinus* (Stewart and Cook, 1998). The variation in dentition patterns observed in *P. warreni* may make it difficult to discern this species from *P. unispinus*. Where populations of the single-toothed *P. warreni* variety and *P. unispinus* occur in close proximity, they may

be considered cryptic species (Daniels *et al.*, 2001b). However, *P. unispinus* always possesses a single, well-defined epibranchial tooth at the epibranchial corner of the carapace and is genetically distinct from *P. warreni*. *P. parvispina* is a species with a small, but distinctive epibranchial tooth at the corner of the anterolateral margins of the carapace and is endemic to high mountain streams of the Western Cape. In *P. bayonianus bayonianus*, *P. b. dubius*, and *P. obesus calcaratus*, the postfrontal crest slopes backward to meet the epibranchial tooth, forming a distinct epibranchial sinus anterior to this tooth, and their distribution does not overlap with that of *P. warreni*.

Results

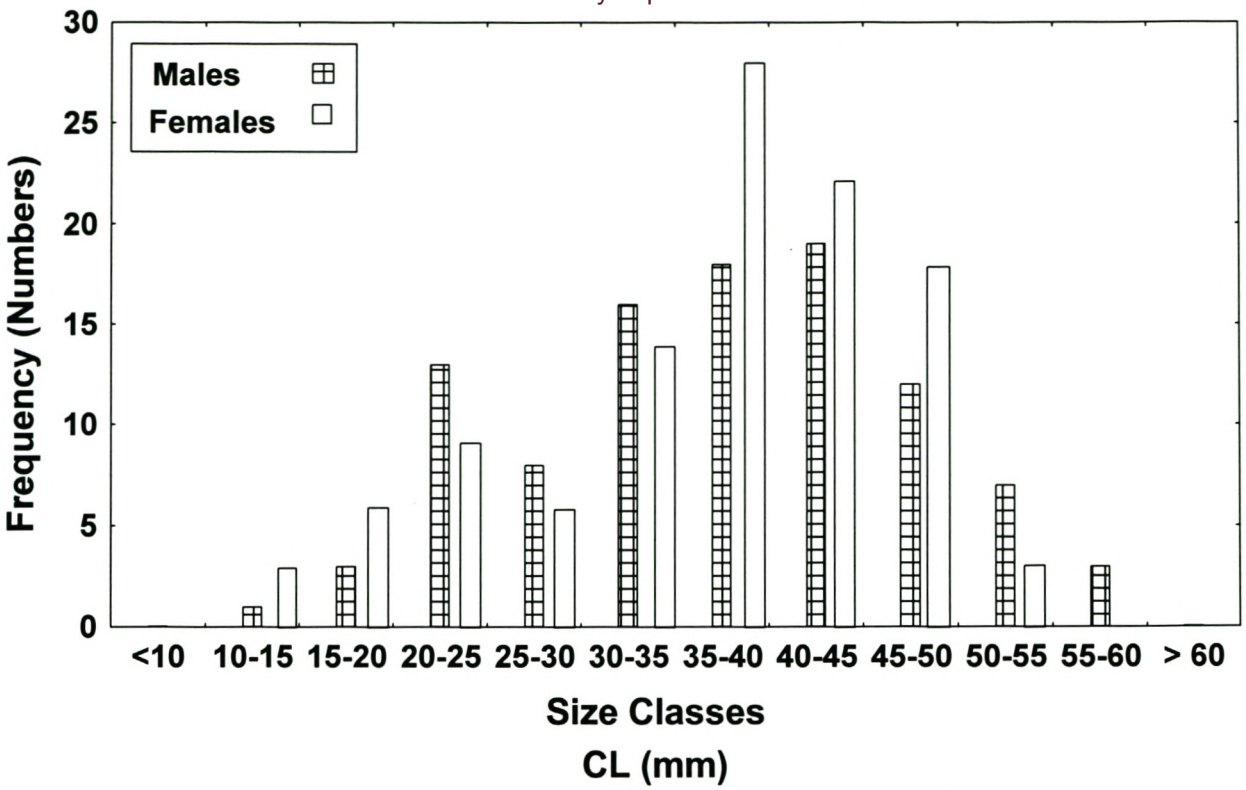
Population structure

Group 1 was represented by 32 males and 45 females, which translated to a sex ratio of 1 : 1.45 (S_0 = -0.16) and an effective population size, Ne = 77; group 2 was represented by 15 males and 13 females which translated to a sex was ratio of 1 : 0.86 (S_0 =0.07) and Ne = 28; group 3 was represented by 71 males and 73 females which correspond to a sex ratio of 1 : 1.02 (S_0 = 0.02) and Ne = 143. Overall, 118 males and 131 females were collected and the sex ratio thus was 1:1.11 (S_0 = -0.05) which did not deviate significantly (χ^2 = 0.04; df =2; $P > 0.05$) from a 1:1 ratio. The estimated effective population size (Ne = 248) was nearly similar to the actual population size (N = 249) due to the high similarity in the overall sex ratio. No significant difference was seen ($P > 0.05$) in the frequency of the carapace length (CL) per size group between sexes (fig. 5. 2). In addition, no significant differences ($P > 0.05$) in the mean size between males and females existed for the following variables, CL, CWW, PFCD, ED, CH, P2PL, P2PW, P2ML, P2MW, P5PL, P5PW, P5ML, P5MW, CLDL,

CLPL, and CLPW. However, when the mean width of the fifth and six abdominal segments was compared between sexes, significant differences ($P < 0.001$) were obtained for both AW5 ($t = -12.61$) and AW6 ($t = -13.43$). Females had significant larger abdominal segments (mean AW5 =23.37 mm; mean AW6 = 24.29 mm) than males (mean AW5 = 9.74 mm; mean AW6 = 11.93 mm). When the mean width of the right dactylus length (CRDL), and the right propodus length (CRPL), and right propodus width (CRPW) were compared between sexes, statistically significant ($P < 0.05$) results were obtained CRDL ($t = 2.42$); CRPL ($t = 2.29$) and CRPW ($t = 2.34$). Males had larger right chelipeds (mean CRDL = 22.03 mm; mean CRPL = 36.07 mm, and CRPW = 14.34 mm) than females (mean CRDL = 19.68; CRPL = 32.42 mm and mean CRPW = 12. 77 mm). A histogram of frequencies of observation for males and females per size class using CL (carapace length) showed no significant differences between size class frequencies for males and females ($P > 0.05$) and between sexes ($P > 0.05$).

Heterochely

Crabs that were missing either cheliped were excluded from the analyses. Of the 208 crabs examined, 17 % (35 of 208) had the left propodus and dactylus enlarged (left handed) and 84 % (175 of 208) had the right propodus and dactylus enlarged (right-handed). No homochelous individuals were found. Among the 100 females sampled, 21% were left-handed and 79% were right handed, while amongst the 105 males examined, 12 % were left-handed and 88 % were right-handed. No significant difference in handedness between sexes was observed ($\chi^2 = 3.80$; $P > 0.05$). Handedness between the sexes was compared relative to CL to assess if any changes



Figuren 5. 2. Histogram of carapace length frequencies for females and males.

occur as animals grow. For females with a CL < 30mm, 68 % (17 of 25) were right handed, while 80 % (20 of 25) males in the same size class were right handed. In the range 30 to 40 mm CL, 77 % (34 of 44) females were right handed, while 95 % (38 of 40) of males were right handed. Of the crabs with a CL > 40 mm, 86 % of females (24 of 28) and 83 % (33 of 40) of males were right handed. Scatterplots of the width of the left and right propodus against their lengths for both sexes show that the right chelae in males and females grow proportionally larger than the left chelae (fig. 5. 3). When the slope of the regression was compared between the right and left propodus length relative to CL within sexes, significant differences between the right and left propodus was evident for both females ($F = 3.99$; $P < 0.05$), and males ($F = 4.69$; $P < 0.05$). In addition, when the regression for the slope of right propodus was compared between sexes statistically significant ($F = 37.05$; $P < 0.001$) results were obtained, with males growing larger in the right propodus length relative to CL (fig. 5. 4).

Allometry

Table 5.2 provides a summary of the allometric statistics for *P. warreni*. The wet weight (WW) of males and females showed positive allometric growth. This demonstrates that the weight of the animal increases as it matures. There was a significant difference in the slope ($F = 7.18$; $P < 0.01$) between log WW and log CL for males and females, due to the small but significant difference in the slope between the two regression lines. The regression of log CWW against log CL showed positive allometry in males, but was negative in females, the slopes between these two variables being significantly different ($F = 17.21$, $P < 0.001$). Both log CWA and log PFCD showed negative allometry for both sexes. Log CWA was significantly

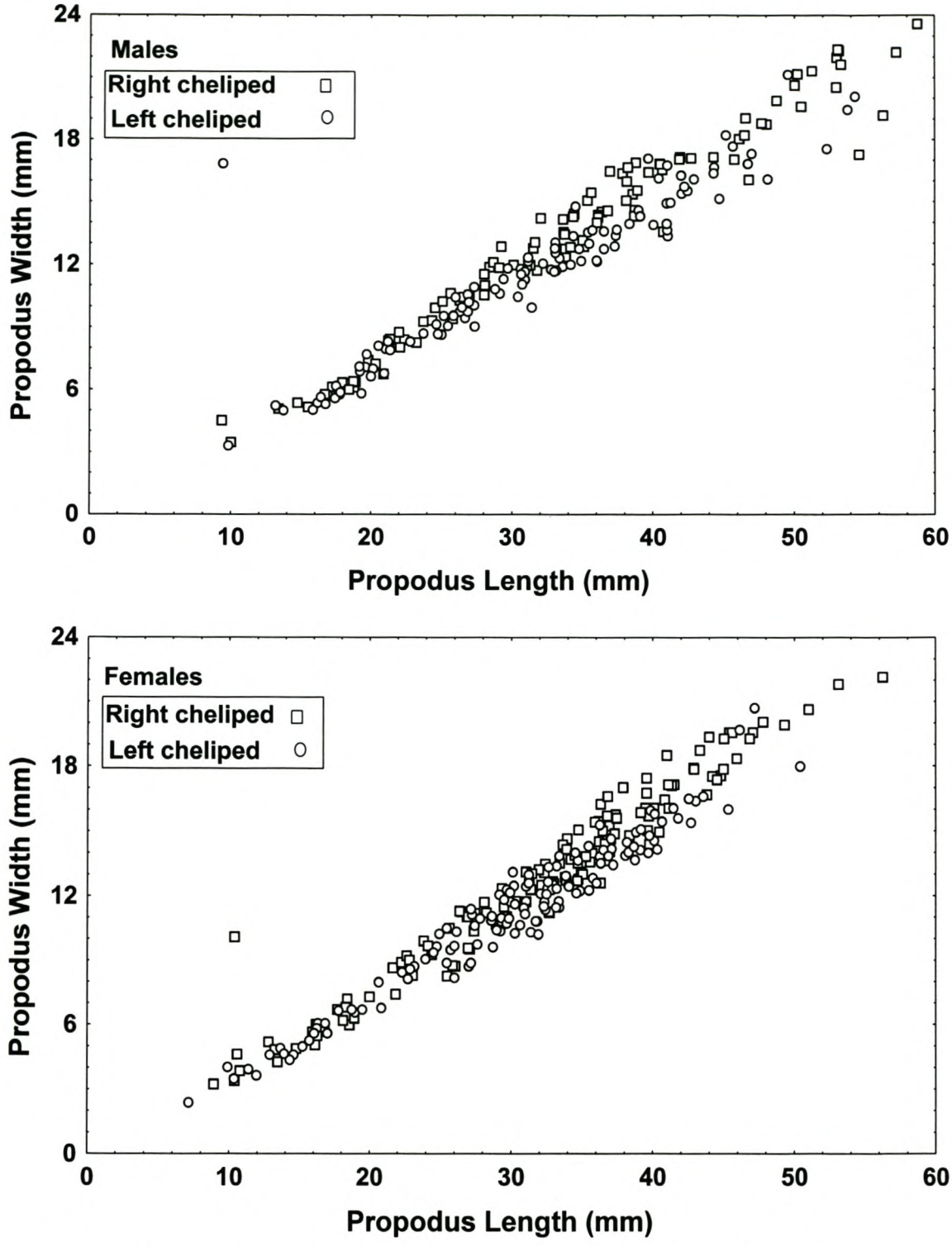


Figure 5. 3. Plot of propodus width against propodus length of the left and right cheliped for male and female specimens.

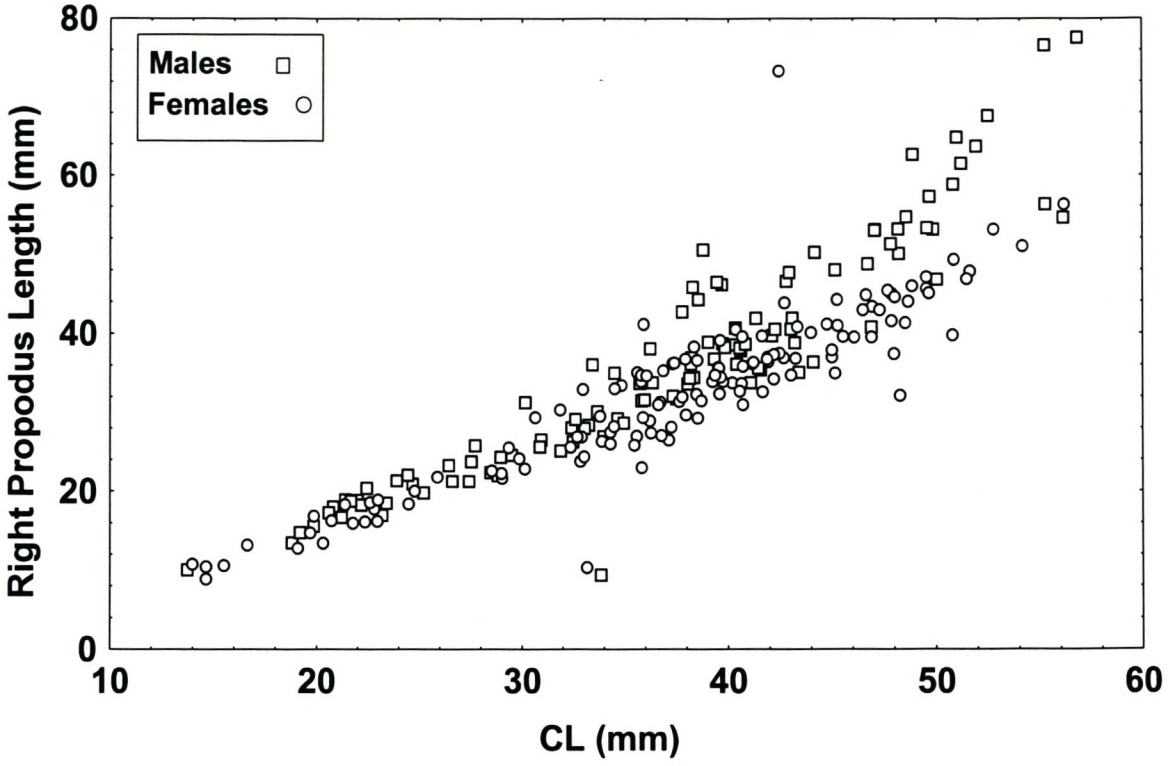


Figure 5. 4. Plot of the right propodus length against carapace length (CL) for males and females.

Table 5. 2. Regression analyses of morphometric data for *P. warreni* (Calman, 1918).

(M = males, F = females; log indicates logarithms of base 10).

X	Y	Sex	Least Square Regression Log Y = log a + b log X	r	$t(b-1)^{(1)}$
CL	WW	M	Log WW = -3.441 + 3.187 (Log CL)	0.995	3.181
		F	Log WW = -3.515 + 3.226 (Log CL)	0.995	3.225*
CL	CWW	M	Log CWW = 0.102 + 1.021 (Log CL)	0.995	1.021*
		F	Log CWW = 0.116 + 1.008 (Log CL)	0.996	0.674*
CL	CWA	M	Log CWA = 0.242 + 0.832 (Log CL)	0.993	0.832*
		F	Log CWA = 0.229 + 0.838 (Log CL)	0.993	0.838*
CL	PFCD	M	Log PFCD = -0.809 + 0.995 (Log CL)	0.973	0.995*
		F	Log PFCD = -0.721 + 0.935 (Log CL)	0.973	0.934*
CL	CH	M	Log CH = -0.578 + 1.199 (Log CL)	0.966	1.198*
		F	Log CH = -0.619 + 1.224 (Log CL)	0.966	1.224*
CL	ED	M	Log ED = -0.224 + 0.982 (Log CL)	0.744	0.982*
		F	Log ED = -0.368 + 1.074 (Log CL)	0.744	1.072*
CL	AW5	M	Log AW5 = -0.4545+ 0.975 (Log CL)	0.975	0.975*
		F	Log AW5 = -1.460 + 1.798 (Log CL)	0.959	1.797*
CL	AW6	M	Log AW6 = -0.644 + 1.039 (Log CL)	0.967	1.038*
		F	Log AW6 = -1.832 + 2.018 (Log CL)	0.955	2.018*

Table 5. 2. continues

X	Y	Sex	Least Square Regression Log Y = log a + b log X	r	t(b-1)[1]
CL	LCPL	M	Log LCPL = -0.464 + 1.249 (Log CL)	0.921	1.249*
		F	Log LCPL = -0.334 + 1.153 (Log CL)	0.977	1.153*
CL	RCPL	M	Log RCPL = -0.603 + 1.369 (Log CL)	0.929	1.398*
		F	Log RCPL = -0.410 + 1.217 (Log CL)	0.932	1.217*
P2PL	P2PW	M	Log P2PW = -0.280 + 0.985 (Log P2PL)	0.952	0.984*
		F	Log P2PW = -0.295 + 1.009 (Log P2PL)	0.960	1.009*
P2ML	P2MW	M	Log P2MW = -0.272 + 0.907 (Log P2ML)	0.940	0.906*
		F	Log P2MW = -0.382 + 1.006 (Log P2ML)	0.950	1.006*
P5PL	P5PW	M	Log P5PL = -0.200 + 0.906(Log P5PW)	0.922	0.906*
		F	Log P5PL = -0.156 + 0.867 (Log P5PW)	0.954	0.867*
P5ML	P5MW	M	Log P5MW = -0.375 + 0.971 (Log P5ML)	0.945	0.971*
		F	Log P5MW = -0.392 + 0.991 (Log P5ML)	0.950	0.990*

(1) t – test for B = 1.

(2) $P < 0.05$ marked with asterisk

different between the two sexes, ($F = 5.78$; $P < 0.05$), for PFCD however no significant differences in the slopes were detected. Log ED against log CL showed negative allometry in males but it was positive in females. No significant difference in slope was evident between males and females for this character. Sexual dimorphism in the both the fifth (AW5) and sixth (AW6) abdominal segment width against CL was evident between sexes. The regression for the log AW5 for males showed negative allometry, while in females it showed positive allometry, and a significant difference in slope was evident between sexes ($F = 8.41$; $P < 0.01$). Log of AW6 against CL was slightly positive in males, and markedly curvilinear, exhibiting positive allometric growth. The regression between these two sexes was significantly different ($F = 901$; $P < 0.001$).

When both the left and right cheliped propodus length (LCPL and RCPL) was compared to CL positive allometry was evident for both sexes, with males showing slightly higher positive values. When the left propodus length against CL was compared between sexes statistically significant results were detected ($F = 8.97$; $P < 0.01$), with males growing slightly faster then females (fig. 5. 5). When the right propodus length against CL was compared between sexes, males grew proportionally larger than females (fig. 5. 6). The slope of this regression between the sexes were statistically significant ($F = 26.95$; $P < 0.001$). Log P2PW against P2PL showed negative allometry for males, but was close to isometry in females, while statistically significant results were obtained between sexes ($F = 4.04$; $P < 0.05$). The regression of P2MW against P2ML showed negative allometry in females, but were close to isometry in males, however no significant differences in the slopes were obtained

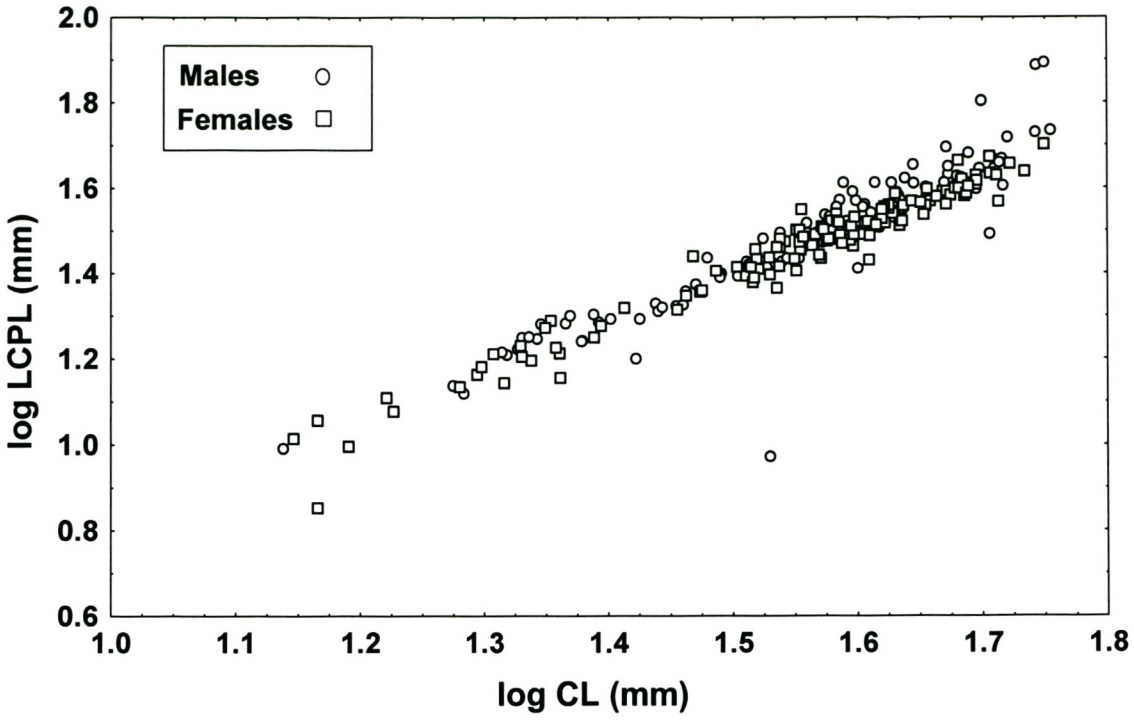


Figure 5. 5. Plot of log left chela propodus length (LCPL) against log carapace length (CL) for males and female *Potamonautes warreni* (Calman, 1918) specimens.

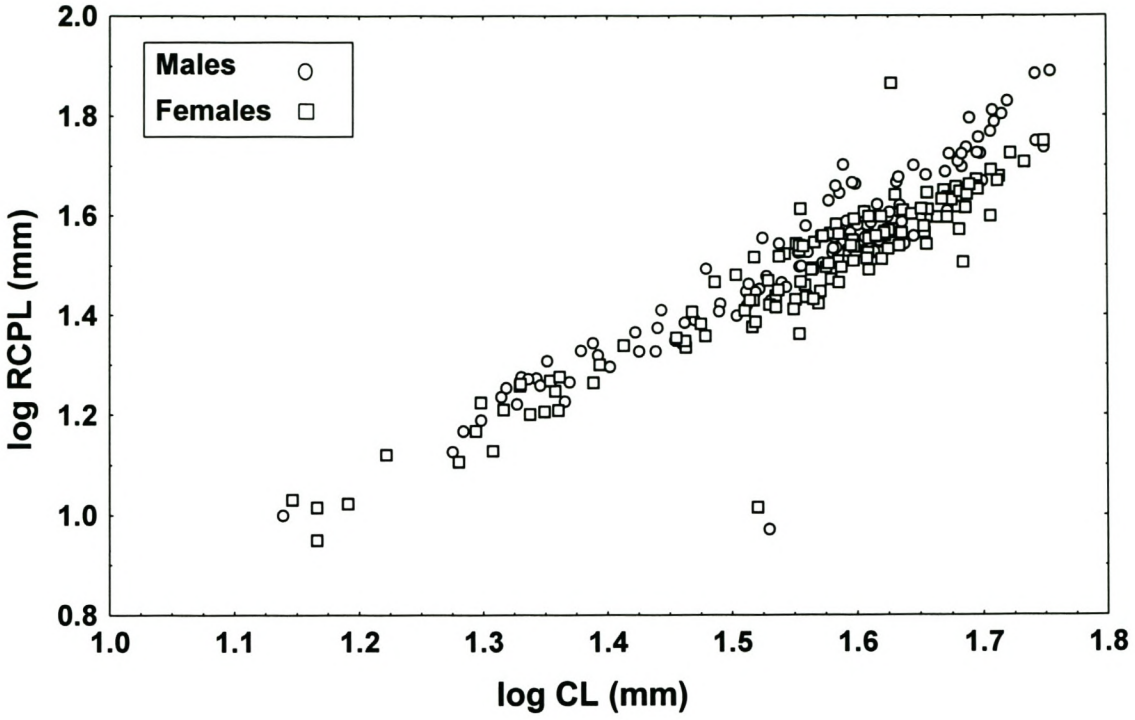


Figure 5. 6. Plot of log right chela propodus length (RCPL) against log carapace length (CL) for males and female *Potamonautes warreni* (Calman, 1918) specimens.

between sexes. Negative allometry was recorded for both sexes for all the measurements of the fifth pereopod, with no significant differences for P5PL against P5PW and for P5ML against P5MW.

Discussion

The sex ratio within the three population groups was close to 1:1. A number of researchers (Raubenheimer, 1986; Cornew, 1990; Mayfield, 1993) reported that female freshwater crabs were the more abundant sex. In this study 52 % of the total number of crabs caught was female. Males and females were generally of similar size. Sexual dimorphism was clearly evident between the abdominal segments, and to a lesser degree in the chelipeds of *Potamonautes warreni*.

The growth of the abdominal width in *P. warreni* showed strong positive allometry and was sexually dimorphic. Hartnoll (1982) described such a general pattern of growth in crustaceans. A number of subsequent studies on sexual dimorphism in decapod crustaceans have reported similar results (Raubenheimer, 1986; Cornew, 1990; Mayfield, 1993). The growth in the male abdomen approaches isometry, while that in females shows strong positive allometry. The sexual dimorphism of the abdomen is thought to be related to the differences in the function of the male gonopods and female pleopods. In male crabs the gonopods need not increase in size faster than the carapace. Finney and Abele (1981) are of the opinion that this does not have any reproductive advantage. In females, however, the marked increase in abdomen size would increase the area for the fixation of eggs on the setose pleopods

and thus act as an incubation chamber for the developing eggs.

Potamonautes warreni is heterochelous, with right handedness in both sexes being the most dominant. Handedness among freshwater crabs in the genus *Potamonautes* (Potamonautidae) is relatively well documented, yet has remained poorly studied (table 5.3). Heterochely showed no sexual dimorphism, as it is equally expressed in both males and females. Similar patterns have also been recorded for freshwater crab of the family Potamidae. For example, Gherardi and Micheli (1989) reported that right handedness in the freshwater crab *Potamon potamios palaestinensis* (Bott, 1967) is dominant. Hartnoll (1982) stated that in the majority of heterochelous decapod species "there is no preference for handedness of the large chela" and "this must be regarded as the prevalent situation, although isolated examples of handedness are recorded". This dominance in handedness is not restricted to freshwater crabs, but has also been reported amongst marine taxa in the Calappidae (e.g. Ng and Tan, 1985); Portunidae (Hamilton *et al.,* 1976; Abby-Kalio and Warner, 1989; Chu, 1999); Xanthidae (Cheung, 1976); and in the Belliidae (Abby-Kalio and Warner, 1989). Even among freshwater crabs not all families are heterochelous. For the African families limited data is available on heterochely.

Most of the studies conducted on handedness in marine crabs have concentrated on the crushing of shells by marine crabs. However, feeding studies in freshwater crabs indicate that these crabs are detritivores or feeding generalists (Hill and O'Keeffe, 1992). These authors showed that juvenile crabs (CL = 21-30 mm) of the Cape River

Table 5. 3. A summary of handedness in the southern African freshwater crabs expressed as a percentage for the sex examined. Where N is the total number of individuals examined per species. The missing percentage was homochelous crabs. R = right-handed, L = left-handed. Unless otherwise stated, data taken from descriptive papers.

Species	Females		Males		N	Reference
	%R	%L	%R	%L		
P. bayonianus (Brito-Capello, 1873)	77	23	92	8	61	Unpub. data
P. brincki (Stewart, 1997a)	58	42	89	11	61	Unpub. data
P. dentatus (Stewart *et al.*, 1995)	82	18	75	25	39	
P. depressus (Krause, 1843)	94	6	94	6	272	Unpub. data
P. clarus (Gouws *et al.*, 2000)	67	7	85	6	75	
P. granularis (Daniels *et al.*, 1998b)	82	15	73	24	113	
P. lividus (Gouws *et al.*, 2001)	74	13	79	8	147	
P. calcaratus (Gordon, 1929)	87	13	88	12	33	Unpub. data

Table 5. 3. continues.

Species	Females		Males		N	Reference
	%R	%L	%R	%L		
P. parvispina (Stewart, 1997b)	61	37	88	12	249	Unpub. data
P. perlatus (H. Milne Edwards, 1837)	79	21	76	24	374	Unpub. data
P. sidneyi (Rathbun, 1904)	71	29	71	29	1432	Raubenheimer (1986)
P. unispinus (Stewart and Cook, 1998)	85	15	79	21	274	Unpub. data
P. warreni (Calman, 1918)	77	23	13	87	201	Present study

crab, *Potamonautes perlatus* (H. Milne Edwards, 1837) ate a significantly higher percentage of aquatic invertebrates than did adults, however as the crab grows (61 - 70 and 71 - 80mm) vascular plant material becomes more abundant. Raubenheimer (1986) reported a similar trend for *P sidneyi*. In freshwater crabs, the enlarged right cheliped may play a less significant role during feeding as crabs usually use the large chelae to grab food and the smaller or minor chelae to feed and manipulate food, and no crushing is required for effective feeding (Daniels, pers. obs.). Cornew (1990) showed an exponential increase in size of the major chelae of *P. perlatus* and suggests that claw dimorphism is related to something other than feeding.

Stein (1976) hypothesized that sexual selection is probably the major driving force in the evolution of large chelae. The larger chela in *P. warreni* may play a role in sexual signalling (pre-mating behavior such as courtship), reproduction (in fighting for females) and in defense (of food, mates or territories). Males with larger chela may have some selective advantage over males with smaller chela, and may thus be more successful at feeding, reproducing and at defending home territories (burrows). Stein (1976) showed that in the crayfish, *Orconectes propinquus* (Girard, 1852), males with larger claws were able to mate with larger females, and that a larger chela determines their superiority during fighting prior to copulation. *Potamonautes warreni* have been observed to burrow extensively (pers obs). Individuals with larger claws may thus be more successful at defending their burrows and territories, assuming freshwater crabs are territorial. Barbaresi *et al.,* (1997) have observed that in the freshwater crab *Potamon fluviatile* (Herbst, 1785) animals occupy a different refuge when they return from their nocturnal feeding. These authors did not report any fights for burrows. However, aggressive behaviour is likely to be influenced by densities.

The large claw of females may act as a signal of sexual vitality and reproductive vigor to males. Freshwater crabs are well known to be direct developers with long incubation periods (for up to three months in *P. sidneyi*) and extensive maternal care (Raubenheimer, 1986). Females with larger claws may thus be more successful at mating and have a better chance of defending the developing young against attack from other crabs. More recently, Liu and Li (2000) showed that in the freshwater crab, *Candidiopotamon rathbunae* (de Man, 1914) pre-and post-hatching maternal care occurs, for up to two weeks. Ovigerous females of this species have been found to prefer to stay on land, to avoid injury due to flash floods. This behavioral adaptation in females freshwater crabs may have led to the evolution of large chelipeds that can be used to defend the developing young against predators and thus enhance the survival rate of juveniles. Ng and Ng (1987) while observing *Somanniathelphusa sexpunctata* that the males use the large claw to embrace the females, providing additional, altbiet marginal support that the enlarged claw has a function related to sexual activities.

Abell'o *et al.*, (1990) reported that isometric growth in the chela of swimming crab would be advantageous, as they would use less energy during swimming. The high positive allometry in both chelae can be related to the fact that freshwater crabs are benthic species, with a reduced swimming capacity. Clayton (1990) noted that caution should be exercised when interpreting patterns of a allometric growth, as the tests that are used to determine if a set of variables exhibit isometric or allometric growth are designed to detect differences, not the absence of differences. He warns that the functional significance of allometric growth has received only superficial treatment and that more care needs to be taken when explaining such biological

phenomena. Cumberlidge (1999a) is of the opinion that the variation in morphometric relationships is correlated with the habitat and lifestyle of freshwater crabs. According to Cumberlidge (1999a), most of these changes in the morphology of freshwater crabs are to accommodate changes in the respiratory system. More research on allometric relationships amongst the South African freshwater crab fauna needs to be undertaken in order to corroborate such conclusions.

Chapter 6

Molecular and morphological data demonstrates the presence of cryptic lineages among freshwater crabs (Decapoda: Potamonautidae: *Potamonautes*) from the Drakensberg Mountains, South Africa

Abstract

The evolutionary importance of cryptic taxa is well documented, yet few studies exist that have examined cryptic species in defining units of conservation. In the present study, the significance of cryptic speciation in freshwater crabs is examined. Nuclear genetic markers (allozymes) and sequence data from the 16 S rRNA region of the mitochondrial genome were used to explore the degree of population differentiation between mountain stream populations of two distinct freshwater crab species. Marked patterns of differentiation were evident among populations based on the allozyme data. In addition, populations that occurred in close geographic proximity were characterized by the fixation of alternate alleles at certain loci, indicating the absence of gene flow among populations. The maternally inherited 16 S rRNA data, mimicked the allozyme results and provide evidence for the recognition of at least five distinct evolutionary lineages separated by pronounced levels of genetic differentiation. Morphometric data on the hand failed to detect any distinct geographically intermediate population groupings. Collectively, the data indicates the presence of five phylogeographic units that are considered cryptic taxa. The conservation priority and the problems associated with cryptic are discussed.

Introduction

A conceptual problem, much debated over the last couple of decades is how and what criteria should be used to define a species (Mayr, 1963, 1970; Templeton, 1989; Cracraft, 1989; Wayne, 1992; Bock, 1992; Avise, 1994). Species are the most fundamental unit in taxonomy, however our understanding of this concept has been plagued by the application of species definitions that are intrinsically impractical when applied to some biological systems (Mayr, 1963,1970; Templeton, 1981, 1989; Cracraft, 1989). Where organisms occur in sympatry defining species boundaries, regardless of which concept is applied, since a single fixed difference (biochemical, genetic, morphological or behavioral) may provide evidence for two separate gene pools. In contrast, allopatric populations may be difficult to define into discreet systematic units, because gene exchange cannot be assessed accurately and partly because such populations may typically be characterized by variability or the absence of diagnostic morphological characters. Assigning organisms to a particular species is further confounded by the occurrence of and sibling cryptic speciation that may only express limited morphological or genetic characteristics to discern taxa.

Cryptic taxa, while today more widespread than previously thought are today considerably more widespread, particularly among invertebrate taxa, such as crustaceans (Knowlton, 1986; 1993). The presence of such ill-defined taxa poses a serious problem for alpha taxonomy as well as conservation authorities. Conservation practices are still largely centered on species directed conservation, however, the occurrence of poorly defined conservation status to endemic cryptic taxa may prove to

be particularly challenging. In an attempt to steer away from species-directed conservation, the use of intraspecific phylogeographic studies has become prominent as it aims to understand and use the historical relationships among populations as a means to assess conservation value (Moritz, 1994). These have led to the recognition of conservation units such as evolutionary significant units (ESU) (Ryder, 1986; Waples, 1991; Moritz, 1994; Karl and Bowen, 1999; Crandall *et al.,* 2000). More recently, Goldstein *et al.,* (2000) warn "conservation decisions should not be based on established taxonomic epithets". The rule of thumb, however, seems to be that a taxon that is not a distinct subspecies or species is not generally worthy of conservation. Consequently, the importance of cryptic taxa in conservation has remained uncharted or largely ignored.

The South African freshwater crab fauna constitutes a rich faunal assemblage. Alpha taxonomic studies on the group have recently led to the description of six new species, many of whom are cryptic taxa including *Potamonautes clarus* (Gouws *et al.*, 2000), described from mountain streams in the northern Drakensberg, within KwaZulu-Natal, South Africa. *P. clarus* has a narrow distribution and lives in close geographic proximity to *P. depressus* (Krauss, 1843), the latter being widely distributed. Genetic studies on South African freshwater crabs has to date revealed that conspecific populations are generally genetic invariant, with only slight changes in allele frequency of polymorphic loci despite marked geographic distances (Daniels *et al.*, 2001a,b). Preliminary allozyme data suggested that population geographically intermediate to either *P. clarus* and *P. depressus* are characterized by fixed allelic differences that differentiate them from either of the parental species, indicating the

possible existence of cryptic intermediate taxa. Subsequently, sampling was conducted between the known southernmost and northernmost distribution limits of *P. clarus* and *P. depressus*, respectively, in an attempt to explore the systematic status of intermediate populations and to extrapolate the use of this data to the management and conservation of these populations. More specifically, we set out to quantify and characterize populations from the geographically intermediate area using morphology, allozymes and sequences data.

Material and methods

Sample collection

The sample sizes of three populations (Oliviershoek, Mahai and Kamberg), for which data were previously published (Gouws *et al.*, 2000), were augmented by additional collection using baited handnets or electrofishing. In addition, six populations were sampled from new localities: Gudu Falls, Cathedral Peak, Doreen Falls, Monks Cowl, Injasuti and Loteni (fig. 6. 1).

Specimens from the Oliviershoek, Mahai and Gudu Falls localities were identified, on the basis of carapace morphology and colouration, as *P. clarus*, while individuals from Loteni and Kamberg were identified as *P. depressus*, using the descriptions of Krauss (1843), Barnard (1950), Bott (1955) and Gouws *et al.,* (2000). Individuals from the geographically intermediate populations (Cathedral Peak, Doreen Falls, Injasuti and Monks Cowl) were found to most closely resemble *P. clarus* in terms of size and carapace shape.

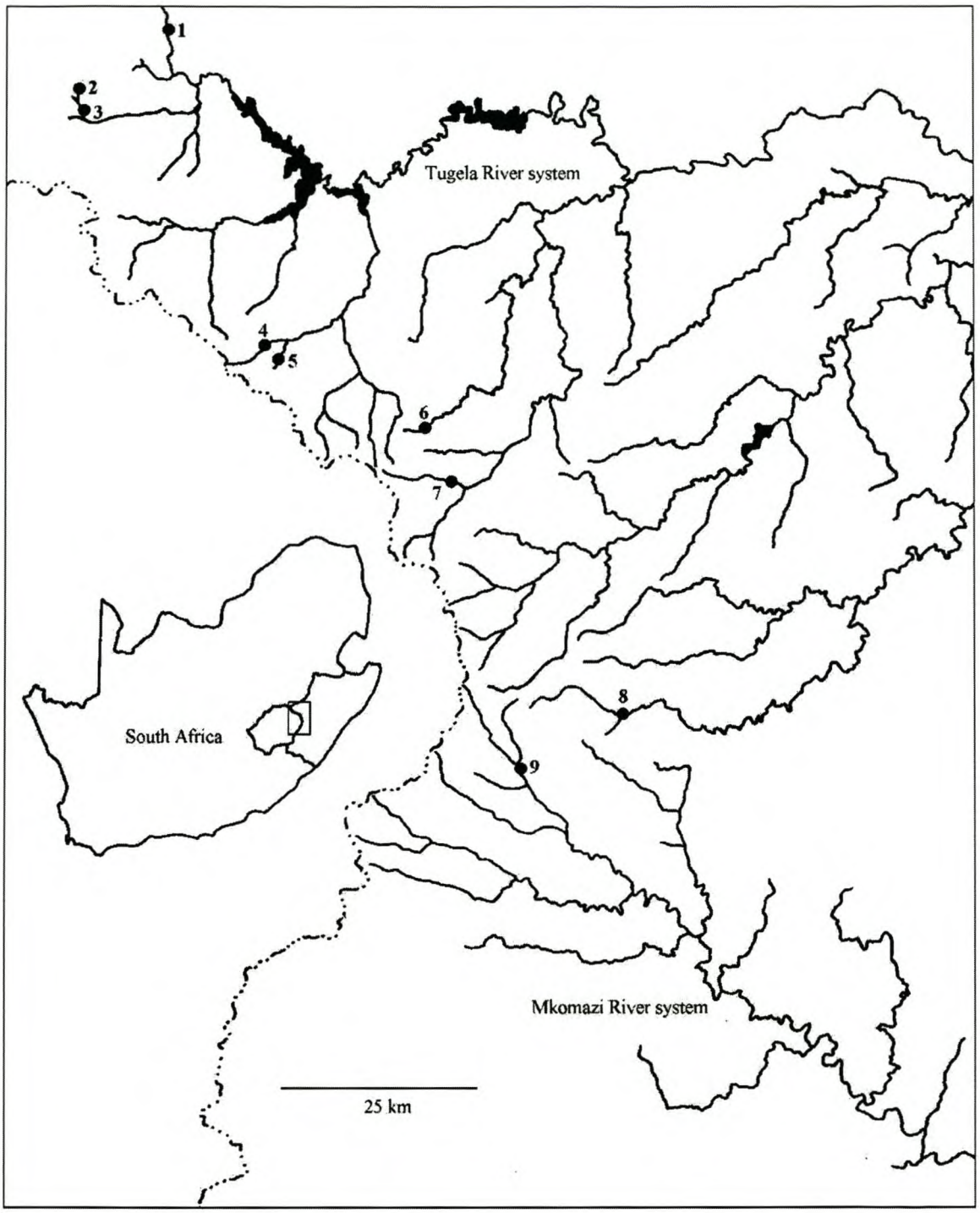


Figure 6. 1. Map depicting sample localities and drainage systems in KwaZulu-Natal, South Africa, (1) Oliviershoek, (2) Gudu Falls, (3) Mahai, (4) Cathedral Peak, (5) Doreen Falls, (6) Monks Cowl, (7) Injasuti, (8) Kamberg and (9) Loteni.

Morphometrics analyses

The measurements used in the present study was similar to those used by Daniels *et al.*, (2001a). These included seven carapace and eight pereopod variables. A discriminate functions analysis (DFA) was performed on to determine whether the populations could be distinguished based on both the carapace and the pereopod variables. These variables were log-transformed and used in the DFA, where specimens were reclassified into one of the populations according to their discriminante functions scores. In addition, a principal component analysis (PCA) of the carapace and pereopod variables was also performed on the log-transformed variables and then subjected to a DFA. Statistical analyses were performed using the computer software programme STATISTICA (Stats Inc., 2000).

Allozyme electrophoresis

Samples were prepared and allozyme electrophoresis performed following the protocol outlined by Gouws *et al.*, (2000). Observed genotype frequencies were compared with genotype frequencies expected under Hardy-Weinberg equilibrium, using a χ^2 goodness-of-fit test and Levene's (1949) correction for small sample size. Genetic variability measures, including the mean number of alleles (A) per locus, percentage of polymorphic loci using no criterion ($P_{\%}$), mean observed heterozygosity (H_O) and mean expected heterozygosity (H_E), were calculated for each population.

Allelic divergence among populations using Weir and Cockerham's (1984) θ-estimate, which is analogous to Wright's F_{ST}. These values, as well as their significance, were calculated for each locus independently and across all loci using the

FStat for Windows V2.9.1 program (Goudet, 2000). Nei's (1978) unbiased genetic identities (*I*) were calculated for pair-wise comparisons among populations and used to construct a dendrogram of genetic similarity, using the UPGMA algorithm (Sneath and Sokal, 1973). A PCA was performed on the allele frequencies of populations using the STATISTICA (Stats Inc., 2000) package. In addition, a discriminant functions analysis was performed on the allele frequencies for each population groups using STATISTICA (Stats Inc., 2000). The jack-knife method was then used to calculate the classification function for each population.

DNA extraction

DNA was extracted from five individuals from each of the nine populations. Total genomic DNA was isolated from 0.05 g of muscle tissue. The tissue was digested in 500 µl of a DNA lysis buffer (200 ml of 1 x STE – 100 mM NaCl, 10 mM Tris-Cl, 1 mM EDTA - and 30 ml of 20 % SDS solution) containing 20 µl proteinase K and 10 µl RNAase. This mixture was incubated for 25-30 min at room temperature and the DNA extracted using standard phenol/chloroform: isopropanol protocols (Hillis *et al.*,1996).

Sequencing

Each reaction tube contained 14.9 µl of millipore water, 3 mM of $MgCl_2$, 2.5 µL of 10 x Mg^2 -free buffer, 0.5 µl of a 10 mM dNTP solution and 0.5 µl of each of the two primers, 16Sar and 16S br (Cunningham *et al.*, 1992) and 0.1 units of Taq polymerase and 3 µl of template DNA. The temperature regime for the polymerase chain reaction (PCR) was 95 ^{0}C for 2 min; 95 ^{0}C for 30 s; 50 ^{0}C for 30 s; 72 ^{0}C for 1 min and then

38 cycles for the last three steps, followed by 1 min at 95 ^{0}C, 3min at 50^{0}C and 15 min at 72 ^{0} C. Samples were electrophoresed in a 1 % regular agarose gel containing ethidium bromide. Products were visualized under UV light. PCR products were purified using a QIAquick PCR purification kit (Qiagen). Purified DNA was cycle sequenced using standard protocols (3 µl of the purified PCR product, 4 µl of the fluorescent-dye terminators (ABI PRISM Dye Terminator Cycle Sequencing Reaction Kit, Perkin Elmer) and 3 µl of a 1 µM of 10mM primer. Unincorporated dideoxynucleotides were removed by matrix filtration using Sepadex G-25 (Sigma). This was followed by analysis on an ABI 377 automated DNA sequencer.

Sequence Analyses

Each sample was sequenced in both directions and checked for base ambiguity in Sequence Navigator (Applied Biosystems). The 16S rRNA sequences were aligned in CLUSTAL W version 1.6 (Thompson *et al.*, 1994) using the default parameters of the program and further adjusted by eye where obvious mismatches were made by the computational alignment. As a result of ambiguity in the first 30 bases of the 16S rRNA gene, this portion was trimmed and excluded from the analysis. Population subdivision was estimated using the Analysis of Molecular Variance (AMOVA) option in ARLEQUIN 1.1 (Schneider *et al.*, 1997); nucleotide and gene diversity was calculated.

Phylogenetic analyses were performed in PAUP*4 version beta 8 (Swofford, 2001) using maximum parsimony (MP), neighbor joining (NJ) and maximum likelihood (ML) methods. In the case of MP analyses, a heuristic search option with random

addition of taxa and with the tree bisection-reconnection (TBR) branch-swapping algorithm was used. For the ML analysis the MODELTEST package, version 3.06 (Posada and Crandall, 1998) was used to determine the best substitution model. For this data set the HKY 85 (Hasegawa *et al.*, 1985) model plus a gamma correction of 0.2282 and a transition transversion ration of 0.8625. The estimates for the parameters of the substitution model obtained for the ML tree was then introduced into the NJ analysis. Statistical support for the nodes were estimated by bootstrapping (Felsenstein, 1985). In this paper, we refer to bootstrap values < 50 % as not supported, bootstrap values between 50 and 70 % as weakly supported and values > 70 % as strongly supported. All trees were rooted using *Potamonautes unispinus* as an outgroup (Daniels unpublished data). Outgroup selection was based on a molecular phylogeny for *Potamonautes*.

Results

Morphometrics

A two-dimensional plot of the individual scores along the first two canonical variables based on the logarithmically transformed carapace measurements for the nine populations showed a clear distinction between the two *P. depressus* populations (Loteni and Kamberg) (fig. 6. 2). The first two canonical variables contributed 88.41% to the total variation among groups. The classification matrix of the nine populations is presented in table 6. 1, and shows moderate support for the designation of populations on morphometric data. In addition, a two-dimensional plot of the individual scores along the first two canonical variables based on the logarithmically transformed pereopods measurements for the nine populations showed some

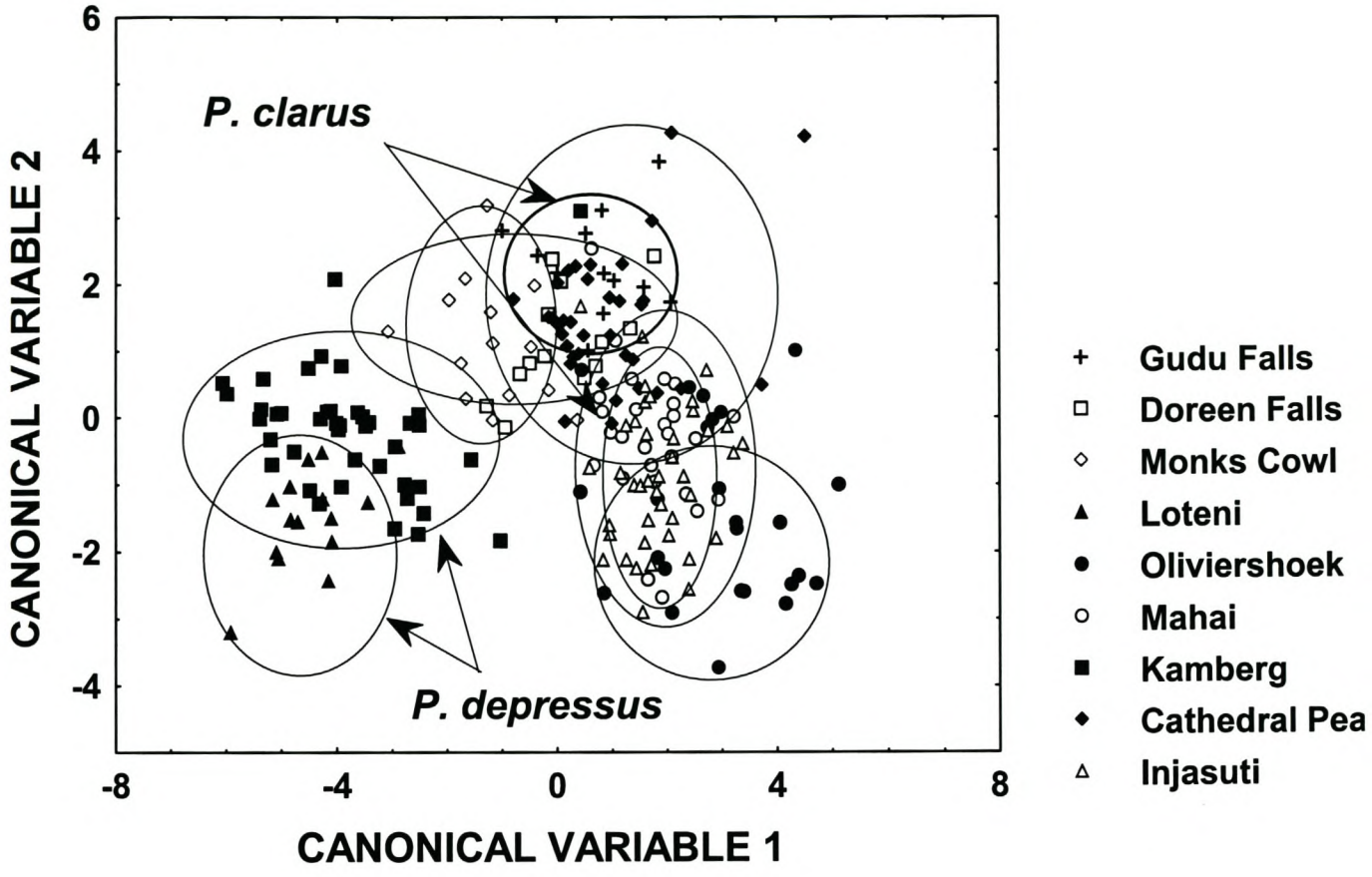


Figure 6. 2. Polygons encompassing the nine population groups based on the logarithmically transformed carapace variables.

Table 6. 1. Percentage correct a posteriori classification to populations on the morphometric classification function of the carapace variables for the nine populations.

	Populations									Correct classification (%)
	1	2	3	4	5	6	7	8	9	
1. Gudu Falls	7	1	0	0	0	3	0	1	0	58.33
2. Doreen Falls	2	4	2	0	0	1	0	0	4	30.76
3. Monks Cowl	1	2	9	0	0	1	0	0	1	64.28
4. Loteni	0	0	0	14	0	0	1	0	0	93.33
5. Oliviershoek	0	0	1	0	12	0	0	4	8	48.00
6. Mahai	1	2	1	0	0	13	0	4	6	48.14
7. Kamberg	0	0	0	1	3	0	37	1	0	88.09
8. Catherdral Peak	0	0	0	0	0	2	0	31	3	86.11
9. Injasuti	0	1	5	0	0	2	0	2	31	75.60

separation, but these groups did not correspond to geographic position or assumed taxonomy. The first two canonical variables contributed 84.13 % to the total variation between populations. The classification matrix for the nine groups as presented in table 6. 2 shows weak support for the correct designation of populations based on the morphometric data, while the morphometric distinction of the two *P. depressus* populations (Kamberg and Letoni) was well supported. The first two principal components extracted for the carapace by the PCA contributed 82.03 % to the total variance between groups. In addition, for the first and second principal component the carapace widest width (CWW) contributed the most to the total variance, while for the third and fourth principal component the distance between the exorbital teeth (CWA) contributed the most. The PCA factor scores corroborated those obtained for the DFA based on the carapace variables and failed to reveal any distinct population groupings (not shown). The first two canonical variables obtained from the analysis of the PCA contributed 89.86 % to the total variation between populations. The reclassification matrix of the populations based on this DFA was generally weak (not shown). The first two principal components extracted by the PCA for the pereopod variables contributed 94.48 % to the total variance among groups. For the first principal component the pereopod 2 propodus width (P2PW) contributed the most to the total variance. In the case of the second principal component the pereopod 5 propodus width (P5PW) contributed the most to the total variance, while for the third and fourth principal component pereopod 5 propodus length (P5PL) contributed the most. The factor scores obtained from the PCA results corroborated those obtained for the DFA based on the pereopod variables, and failed to reveal any distinct population groupings (not shown). The first two canonical variables obtained from the analysis

Table 6. 2. Percentage correct a posteriori classification to populations on the morphometric classification function of the pereopod variables for the nine populations.

	Populations									Correct classification (%)
	1	2	3	4	5	6	7	8	9	
1. Gudu Falls	5	0	2	0	1	3	0	0	1	41.66
2. Doreen Falls	1	4	1	0	0	2	0	2	3	30.76
3. Monks Cowl	0	0	7	0	1	2	0	0	4	50.00
4. Loteni	0	0	0	5	0	0	9	0	0	35.17
5. Oliviershoek	0	1	1	0	15	2	0	0	1	75.00
6. Mahai	2	0	1	0	2	17	0	0	5	62.96
7. Kamberg	1	0	0	0	0	0	27	0	3	87.09
8. Catherdral Peak	0	0	0	0	0	0	0	36	0	100.00
9. Injasuti	1	1	3	0	1	4	0	0	30	75.00

of the PCA contributed 90.0 % to the total variation among populations. The reclassification matrix of the populations based on the DFA was generally weak (table not shown).

Allozyme analyses

Of the 16 loci assayed, two (*Mpi* and *Pgm-2*) were monomorphic across all populations. Allele frequencies at the fourteen polymorphic loci and genetic variability measures are tabulated in Appendix 3. At least two loci were polymorphic in each of the populations, with the percentage of polymorphic loci ranging from 12.5 % to 56.3 %. The mean number of alleles per locus varied from 1.2 to 1.6. Mean observed heterozygosity varied between 0.016 and 0.063, with expected heterozygosity ranging from 0.033 to 0.091 (table 6. 3).

Of the 51 cases of polymorphism, involving all loci and all populations, 26 (50.98 %) show genotype frequencies which deviated significantly from those expected under Hardy-Weinberg equilibrium. Examination of individual multilocus genotypes revealed that collections from both the Cathedral Peak and Doreen Falls localities contained two sympatric gene pools. Individuals were simultaneously fixed for alternate sets of alleles at the *Pep-GL*, *Pep-D*, and *Pgm-1* loci in the case of the Cathedral Peak sample, and the *Idh-2*, *Mdh-2*, *Pep-D*, *Pgd*, and *Pgm-1* loci in the Doreen Falls sample, with these loci not conforming to Hardy-Weinberg expectations. These two populations were split (Cathedral Peak A and B, and Doreen Falls A and B, respectively) and considered independently in subsequent analyses. All deviations were due to a deficiency of heterozygous individuals.

Table 6. 3. The mean number of alleles per locus, the percentage of the loci that were polymorphic and the mean heterozygosity among the populations. Observed heterozygosity (H_O), expected heterozygosity (H_E).

Population	Mean sample size	Mean no. of alleles per locus	Percentage polymorphic	H_O	H_E
1. Oliviershoek	34.9	1.2	12.5	0.020	0.039
2. Gudu falls	15.9	1.4	37.5	0.016	0.500
3. Mahai	47.9	1.3	25.0	0.024	0.033
4. Cathedral Peak A	25.7	1.3	25.0	0.040	0.061
5. Doreen Falls A	3.0	1.2	12.5	0.063	0.083
6. Cathedral Peak B	3.9	1.2	18.8	0.036	0.070
7. Doreen Falls B	26.9	1.6	43.8	0.048	0.077
8. Monks Cowl	14.9	1.4	25.0	0.029	0.078
9. Injasuti	42.4	1.6	56.3	0.036	0.055
10. Loteni	15.0	1.3	25.0	0.042	0.046
11. Kamberg	29.9	1.4	37.5	0.039	0.091

Substantial genetic sub-structuring among populations was evident from Weir and Cockerham's (1984) θ-estimates across all loci (0.817). Values for individual loci varied between 0.042 (*Me-1*) and 0.985 (*Ark-1*). With exception of the *Idh-1*, all values obtained were significant (table. 6. 4). These data show that there is significant genetic differentiation among the populations sampled.

The dendrogram (fig. 6. 3) constructed using Nei's (1978) genetic identity (*I*) showed two distinct clusters, separated at a *I*-value of 0.557. The populations identified as *P. clarus* (Oliviershoek, Mahai and Gudu Falls) and *P. depressus* (Loteni and Kamberg) formed two distinct groups within the larger clusters. Identity-values varied between 0.976 and 1.000 in the *P. clarus* populations, while an *I*-value of 0.943 separated the two *P. depressus* populations. Interspecific comparisons yielded *I* values of 0.534 - 0.568, comparable to the *I* value (0.599) obtained in the original delimitation of *P. clarus* from *P. depressus* (Gouws *et al.,* 2000). Of the remaining, geographically intermediate, populations, the Cathedral Peak A, Doreen Falls A and Cathedral Peak B populations formed a cluster with the *P. clarus* populations, while the Doreen Falls B, Monks Cowl and Injasuti populations clustered with the two *P. depressus* populations. Comparisons of these intermediate populations with the *P. clarus*, and *P. depressus* populations yielded *I* values of 0.480 - 0.852, and 0.578 - 0.838, respectively. *I* values among geographically intermediate populations resulted in *I* values of 0.527 - 0.994. Interestingly, the most divergent populations within this intermediate group were Doreen Falls A and Doreen Falls B populations, although the small sample size for the Doreen Falls A has probably biases this result. Generally, the dendrogram, with the exception of the placement of the Cathedral Peak A and

Table 6. 4. Summary of the F- statistics over the polymorphic loci over all populations. $F_{(ST)}$ values that are significant are marked (*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$). Unmarked values are not significant.

Locus	$F_{(IS)}$	$F_{(IT)}$	$F_{(ST)}$
Ark-1	-0.035	0.984	0.985***
Idh-2	-0.077	0.958	0.961**
Pep Lt-1	0.801	0.833	0.161*
Gpi-1	-0.017	0.224	0.237**
Me-1	0.476	0.498	0.042
Hex-1	0.167	0.474	0.369**
Mdh-1	1.000	1.000	0.962**
Mdh-2	-0.059	0.963	0.965***
Idh-1	0.416	0.728	0.535**
Ldh-1	0.474	0.498	0.046
Pgm-1	0.663	0.988	0.966*
Pep Gl-1	0.379	0.693	0.506**
Pgd-1	0.596	0.973	0.933*
Pep D-1	0.860	0.997	0.977*
Mean	0.390	0.889	0.817***

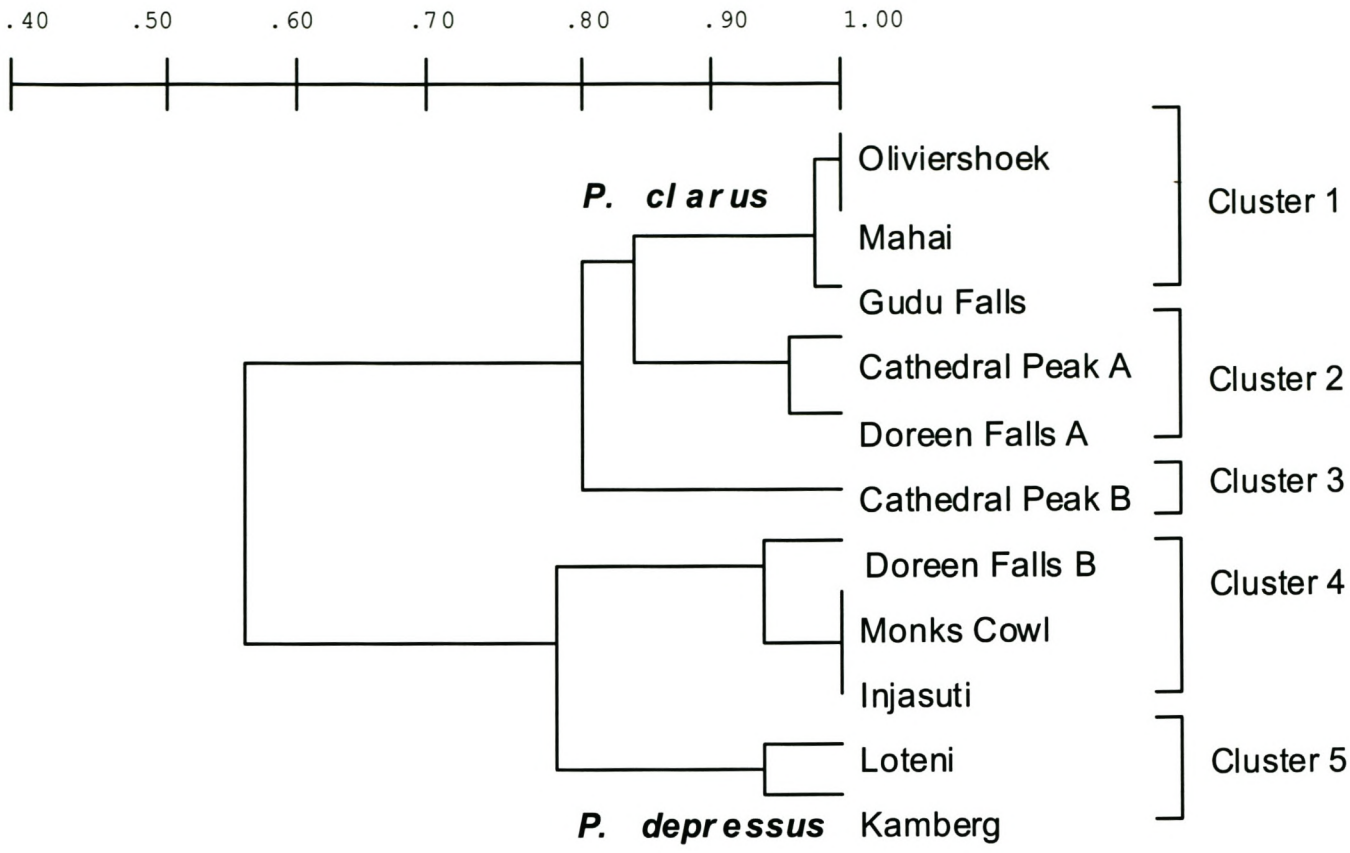


Figure 6. 3. Distance matrix derived from the allozyme data.

Doreen Falls A populations, reflects the geographic distribution of the populations.

The geographic groupings obtained from the PCA (fig. 6. 4) based on the allele frequencies were largely congruent with those obtained from the UPGMA-dendrogram. Four distinct groups were evident, comprised of the species, *P. depressus* (Kamberg and Loteni) and *P. clarus* (Gudu Falls, Oliviershoek and Mahai) and two intermediate groups. In addition, there is an abrupt changes in allele frequency, near-fixed, and fixed allelic differences at many loci (e.g. *Ark*, *Idh-2*, *Mdh-1*, *Mdh-2*, *Pep-GL*, *Pep-D*, *Pgd* and *Pgm-1*, and to a lesser extent *Hk* and *Idh-1*). Where near-fixed allele differences are seen, heterozygous individuals or individuals homozygous for the alternate alleles are often present in populations geographically removed from these allele frequency boundaries. These incongruencies and allelic differences, particularly at the *Ark*, *HK*, *Idh-2*, *Mdh-1*, *Mdh-2*, *Pep-D*, *Pep-GL*, *Pgd*, and *Pgm* loci, define five ostensibly genetically independent populations or population groups. These include: a *P. clarus* group (Oliviershoek, Gudu Falls and Mahai), a group formed by the Cathedral A and Doreen Falls A, a group formed by the Cathedral B population, a group comprising the Doreen Falls B, Monks Cowl and Injasuti populations, a group comprising the *P. depressus* group (Loteni and Kamberg). The discriminant functions analysis of the allele frequencies did not show any distinct population groupings. The classification function of the populations groups revealed a large degree of similarity, particularly among the two know species, *P. clarus* and *P. depressus*. For both these two species, the classification of individuals to a into populations with the highest posterior probability ranged from as low as 25% (Gudu Falls), 40% (Oliviershoek) to as high as 89% (Mahai) for *P. clarus* while in *P.*

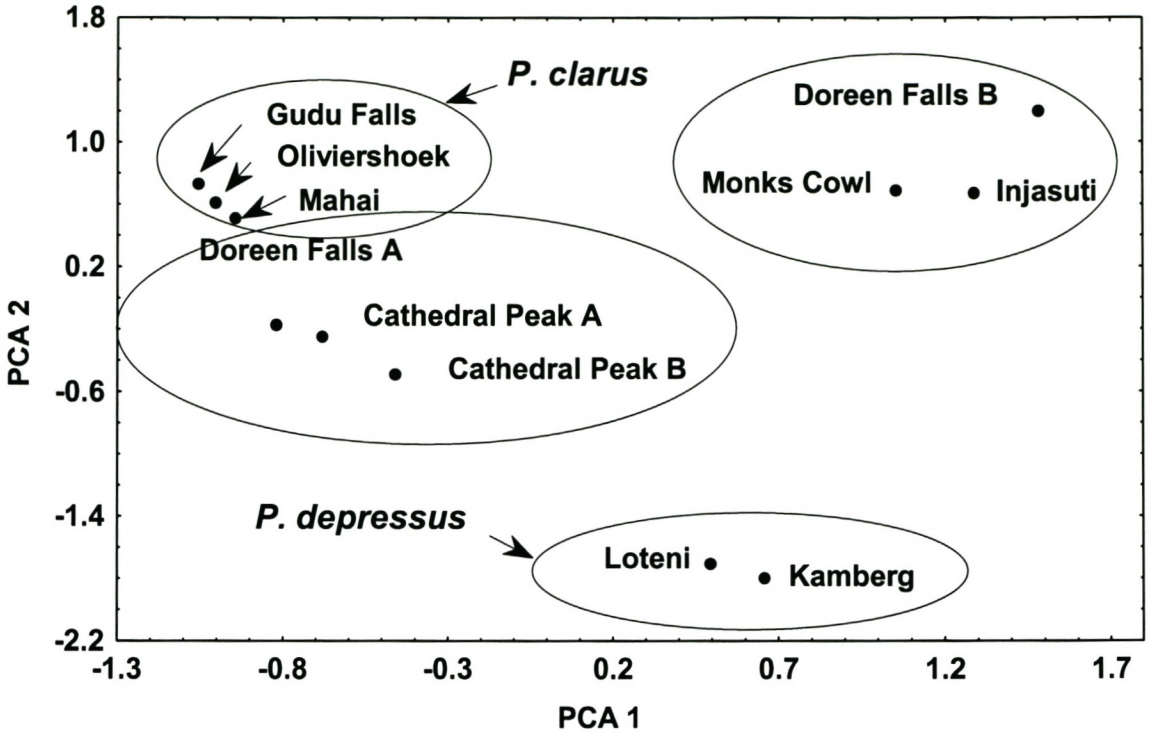


Figure 6. 4. Principal component analysis (PCA) of allele frequencies for the population groups.

depressus the values ranged from 13% (Loteni) to 87% (Kamberg). While these values are low, it is interesting to note that all the re-assigned individuals belonged to other populations of the same species. These results suggest that individuals that belong to these two species can readily be distinguished from all other taxa. For the geographically intermediate groups the reclassification ranged from 56% in Injasuti, 67% in Doreen Falls A, 75% in Doreen Falls B, 96% in Cathedral Peak A to 100% in both Cathedral Peak B and Monks Cowl. This high percentage of correct classification indicates that these geographically intermediate populations are genetically distinct.

Sequencing

A 454 base pair fragment of the 16 S rRNA mt DNA gene was amplified and sequenced from 45 specimens from the 11populations. Sequences have been deposited in GenBank (AY042275 - AY042319). Compositional bias with unequal proportions of the bases is characteristic in most mt DNA studies. All specimens exhibited a high proportion of A and T (38.77 and 35.94 % respectively) while lower proportions of G and C were found (15.62 and 9.65 % respectively). Of the 45 specimens sequenced, a total of 32 haplotypes were identified. The number of haplotypes within a single population ranged from one to five. However, it is difficult to make accurate assessments between localities because of the low number of individuals sequenced. AMOVA indicated that among population variation (Va) was at 19.37 % ($P > 0.05$). In addition, the total variation within populations (Vb) was at 80.63 % ($P > 0.05$). The nucleotide diversity ranged from 0 to 0.016 while the gene diversity ranged from 0 to 1. The corrected sequence divergence values ranged from 0

to 5 % within populations, while the divergence between populations ranged from 2 to 10 %.

Phylogenetic analysis using three algorithms resulted in largely congruent tree topologies. Unweighted maximum parsimony analysis of the haplotypes produced 30 minimal-length trees with a CI =of 0.72 and a RI of 0.93. The bootstrapped parsimony tree showed five distinct clades (fig. 6. 5). Clade one comprised of individuals from Cathedral Peak and Doreen Falls, and formed the sister clade to four populations (clade two) that included all the individuals from Gudu Falls, Mahai and Oliviershoek Pass. The latter individuals could all be identified based on morphology as belonging to *Potamonautes clarus*. Clade three comprised of individuals from Monks Cowl, while clade four comprised of individuals from the Kamberg and Loteni populations of *P. depressus*. Clade five comprised of individuals from Doreen Falls, Injasuti and Cathedral Peak. Bootstrap support for the sister taxon relationship between clade 1 and clade 2 was moderate (61 %), while support for clades 2 and 3 was strong (77 %); similarly, bootstrap support for clades 4 and 5 was strong > 75 %. The terminal tips of the tree were generally poorly supported. The topology of the NJ tree and ML tree supported the same five monophyletic groupings as those obtained using MP. Interestingly, specimens from Doreen Falls and Cathedral Peak fell into two discreet clades, lending support to the idea that at least two distinct gene pools are present at these localities.

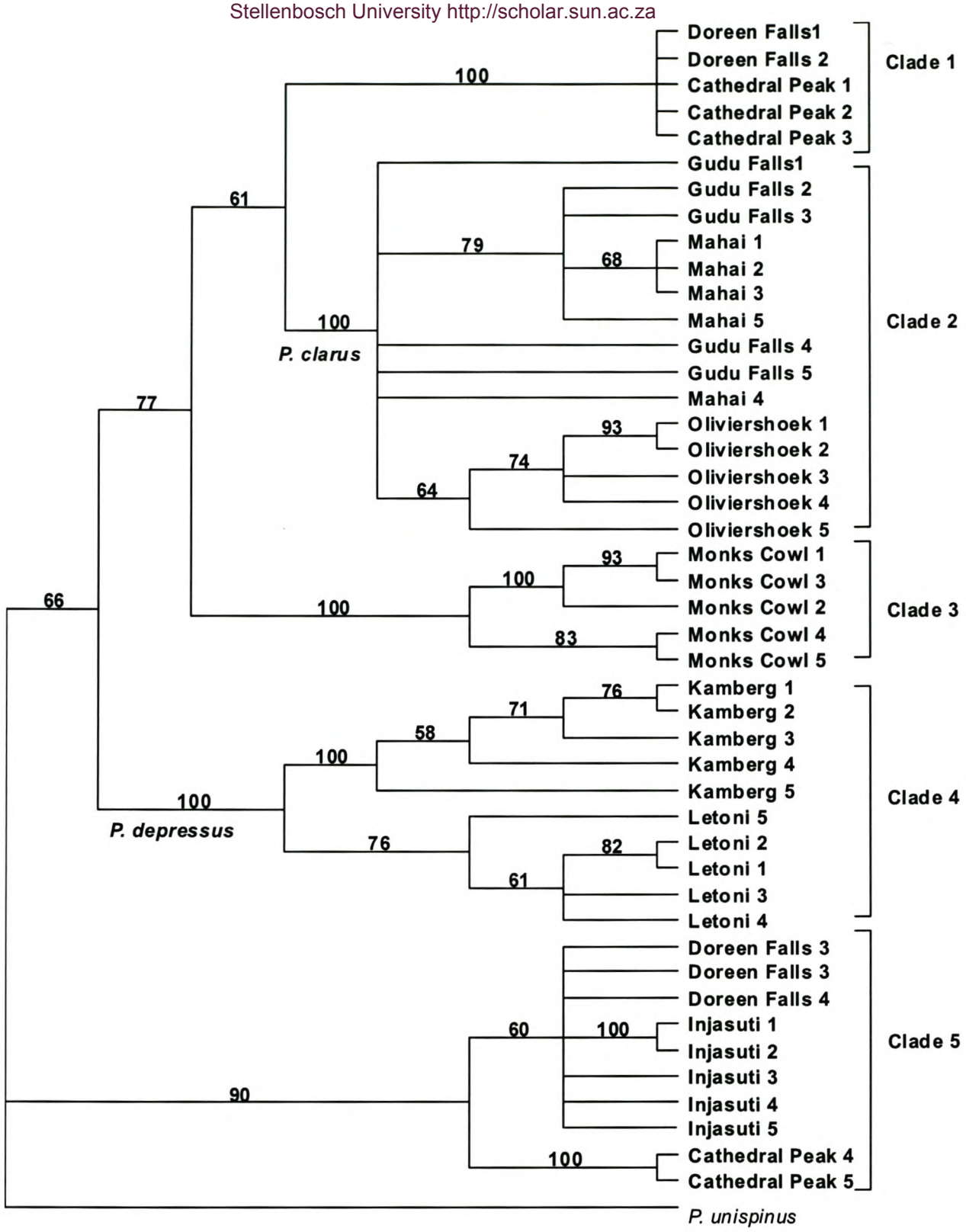


Figure 6. 5. A MP tree with bootstrap support values for nodes on the tree, bootstrap values < 50 % are not shown.

Discussion

Overall, the genetic markers revealed a large degree of congruence and generally support the genetic distinctiveness of the same groups, while the morphometric data failed to detect distinct population groupings particularly for the intermediate and *P. clarus* populations (table 6.5). Tree building methods for the sequence and the allozyme data supported the presence of two gene pools at the Cathedral Peak and Doreen Falls populations, thus validating their split into two discreet populations. Furthermore, both genetic markers retrieved the relationship between the *P. depressus* and *P. clarus* populations, as well as the relationship between Doreen Falls A and Cathedral Peak A. Discordant patterns was however evident. The allozyme phenogram placed Cathedral Peak B as a distinct cluster, while in the sequence data this population was placed as a sister taxa to Injasuti and Doreen Falls B. Similarly, the allozyme data placed Monks Cowl as a sister taxa to Injasuti and Doreen Falls B, while the sequence data placed Monks Cowl was placed as a sister taxon to the *P. clarus* populations. These conflicting results imply that different selection pressures may be operational on the allozyme and mitochondrial genome.

Noteworthy is the fact that the six populations (Cathedral Peak A and B, Doreen Falls A and B, Monks Cowl and Injasuti) from the zone that is geographically intermediate to the two parental taxa are on a unique evolutionary trajectory, as evident by the relative low genetic identities, fixed allele differences and the high sequence divergence values. The genetic *I* values reported here (< 0.85) among the five putative taxa compares favorably with those reported between congeneric freshwater crab species, as well as genetic *I* values reported for a range of invertebrates (Daniels

Table 6. 5. Groups recovered with allozymes and mt 16 S rRNA sequence data for the 11 freshwater crab populations.

Population	Cluster recovered with allozymes	Cluster recovered with sequencing	Congruent
Oliviershoek	Cluster 1	Clade 2	Yes
Mahai	Cluster 1	Clade 2	Yes
Gudu Falls	Cluster 1	Clade 2	Yes
Cathedral Peak A	Cluster 2	Clade 2	Yes
Doreen Falls A	Cluster 2	Clade 2	Yes
Loteni	Cluster 5	Clade 4	Yes
Kamberg	Cluster 5	Clade 4	Yes
Monks Cowl	Cluster 4	Clade 3	No
Doreen Falls B	Cluster 4	Clade 5	No
Injasuti	Cluster 4	Clade 5	No
Cathedral Peak B	Cluster 3	Clade 5	No

et al, 2001a, b). In addition, the presence of pronounced genetic heterogeneity and fixed allelic differences between populations indicate that they should be considered genetically isolated as they have evolved into distinct cryptic lineages.

Population differentiation using morphometric data

The resolution by the morphometric data was poor, indicating that such data have limited application in designating historical biogeographical units when dealing with cryptic taxa. However, the two parental taxa could be easily discriminated when the intermediate populations were excluded from the analysis. The morphological similarity of the intermediate populations in terms of size and carapace shape, is not surprising particularly when dealing with cryptic taxa. The poor discrimination of the cryptic is likely to be confounded by the high degree of phenotypic plasticity. For example, colour differences are well pronounced between *P. depressus* and *P. clarus*, with the later being orange and the former dark brown, while geographically intermediate populations possessed a mixture of these colour features as well as novel colour changes that ranged from wine red to yellow. Among crustaceans, variation in colour has been viewed as useful as a taxonomically diagnostic character for cryptic species (Knowlton, 1986). Such differences in colour can often be complimented by the use of additional data sets.

Population structure

Owing to the presence of various abrupt changes in allele frequency, fixed and near fixed allele differences, it is contended that each grouping, too a large extent, is genetically isolated. The population groups (Cathedral Peak A - Doreen Falls A,

Cathedral Peak B, Doreen Falls B, Monks Cowl - Injasuti, respectively) geographically intermediate to the *P. clarus* and *P. depressus* populations present evidence suggestive of a historical isolation. Genetic variability measures in this study generally appeared to be higher in the intermediate populations, with the highest proportion of polymorphic loci ($P_{\%}$), largest number of alleles (A) and highest observed heterozygosities (H_O) being recorded in populations within the intermediate group. Considering that individual populations appear to exhibit unique, characteristic multi-locus genotypes, often distinct from geographically proximate populations, deviations from Hardy-Weinberg expectations due to a deficiency of heterozygous individuals may result from the detection of two genetic samples, perhaps reflecting the presence of migrants from adjacent, distinct populations. The high degree of reclassification of individual based on the allele frequencies clearly indicate that the geographically intermediate populations are as genetically distinct as the two known.

The geographically intermediate cryptic taxa probably arose through isolation of a widespread ancesteral species. Subsequent isolation and drift of allele frequencies in these intermediate populations would cause the fixation of alleles and result in marked genetic differentiation between allopatric populations. The degree of genetic differentiation between the two parental taxa, the absence of obvious F_1 hybrids or backcrossed progeny leads to the conclusion that the geographically intermediate zone has stabilized historically, with the characters inherited from divergent lineages being retained and transmitted from parent to offspring.

Sequence divergence and phylogeographic structure

Pronounced genetic differentiation was evident among the five population groups, and the relationships between groups were generally well supported by bootstrap analyses, further corroborating the distinct cryptic taxa. Considerable sequence divergence was evident between the clades supporting their genetic distinctiveness. These sequence divergence values are higher than those reported between congeneric decapod species for the same gene fragment. For example, Schubart *et al.,* (1998a) reported sequence divergence values ranging between 2.1 and 2.7 % for *Sesarma* species, while he reported that intraspecifc variation was < 1 %. Similarly, Geller *et al.,* (1997) reported values between 2.5 - 3.9 % between *Carcinus* species while the sequence divergence values between two *Menippe* species was 2.1 %. 16 S rRNA sequence divergence data for potamonautid freshwater crab species indicates that divergence values can be quite variable and range from as low as 1 % between sibling species to as high as 23 % between distantly related species. Interestingly, the sequence divergence values between other pairs of hybridizing freshwater crab taxa are generally < 5 %. The high sequence divergence values reported for such a small geographic area in the present study exceeds that of most interspecific comparison and are indicative of strong geographic subdivision, long temporal isolation and limited dispersal capabilities. Sequence divergence values should be used with caution to indicate reproductive or genetic isolation, as it would appear the organisms may be reproductively compatible despite marked genetic divergence. For example species of *Daphnia* can still hybridize despite exhibiting high sequence divergence up to 19.2 % (Schwenk *et al.,* 2000).

Durand *et al.,* (2000) proposed that high genetic divergences between lineages are indicative of large historic population sizes. However, in the present study the low nucleotide diversity and the occurrence of shared haplotypes between populations does not corroborate such conclusions, instead it is more likely that populations have undergone historic fluctuations. Allopatric divergence appears to be a very important factor in the formation of these taxa. In the absence of fossil data for this group with which to test biogeographic hypothesis, it is difficult to calibrate rates of molecular evolution. Instead, a molecular clock calibrated from 16 S rRNA sequence data derived from decapods has shown that a sequence divergence of 0.9 to 0.88 % occurs per Myr (Strumbauer *et al.,* 1996; Schubart *et al.,* 1998b). When this molecular clock is applied to our data, it becomes evident that the major cladogenic events took place during the late Miocene and early Pliocene (Deacon, 1985). Data on the Miocene /Pliocene climatic changes in the area is not available, however climatic change from other regions in South Africa provides corroborative evidence for major climatic occilations during this time period that would undoubtedly affect the expansion and contraction of distribution ranges of species. Such scenarios provide the ideal grounds for speciation to occur and probably played a major role in the diversification of these freshwater crab populations.

Conservation and ESU's

The degree of genetic differentiation detected in the mtDNA gene tree suggests long-term matrilineal historic genetic isolation that coincides with five crypic taxa. However, mtDNA lineages may not accurately reflect organismal phylogeny because of differences in male and female mediated gene flow, lower effective number of

genes and random lineage sorting (Avise, 1995; Moritz, 1994). Such concern has led to the suggestion that these data be supported with additional information from independent markers such as nuclear loci. In the present study the allozyme data were congruent with the mtDNA sequence data, thus supporting the recognition of five phylogeographic units, or evolutionary significant units (ESU's) (table 6.5). How do the results of the present study fit into the ESU concept?

Ryder (1986) proposed that "populations that actually represent significant adaptive variation based on concordance between sets of data derived from different techniques" should be considered ESU's. Adaptive significance has always been controversial to demonstrate practically, thus this definition has largely been replaced by those advocated by Waples (1991) and Moritz (1994). Waples (1991, 1995) defined an ESU as "a population or group of populations that (1) is substantially reproductively isolated (or at least geographically fragmented) from other conspecific populations and (2) represents an important component in the evolutionary legacy of a species". Considering that fixed allelic differences (revealed by both markers) and large genetic distances exist between the groups, evidence would suggest that the five groups are indeed at present reproductively isolated, thus satisfying the first component of this definition. Noticeably the second term is more difficult to define, hence, Waples (1995) offers the definition that the "the evolutionary legacy of a species is the genetic variability that is a product of past evolutionary events and that represents the reservoir upon which future evolutionary potential depends". The marked genetic difference present between the five-phylogeographic units satisfies this second criterion.

Moritz (1994) argues, "ESU's should be reciprocally monophyletic for mt DNA alleles and show significant divergence of alleles at nuclear loci". When this definition is applied to our results, five phylogeographic units can be defined based on the allozymes (nuclear markers) and mt DNA sequence data, and these are characterized by pronounced genetic divergence and fixed genetic differences, thus constituting five ESU's. Crandall *et al.*, (2000) argues that the ESU concept should incorporate ecological and genetic variation of adaptive significance. These authors define ecological exchangeability as "the factors that define the fundamental niche and the limits of spread of new genetic variants through genetic drift and natural selection" while genetic exchangeability is defined as " the factors that define the limits of spread of new genetic variants through gene flow. These authors further propose that ecological exchangeability should be rejected " when there is evidence for population differentiation owing to genetic drift or natural selection". Genetic exchangeability is rejected when there is evidence of restricted gene flow between populations. If these criteria are applied to the results from the present study, the genetic exchangeability can be rejected on both a contemporary and historic time scale while the ecological criteria can be rejected on a contemporary but not historic time scale. The authors propose a number of scenarios to aid in the recognition of ESU's for management. The population groups representing the five-phylogeographic units should be regarded as distinct ESU's and management action should aim to preserve the adaptive divergence and evolutionary distinctiveness of these populations, regardless of their hybrid origin. Herein however lies a paradox, as the ESA (Endangered Species Act) of the USA for example does not offer any legal protection for invertebrate populations unless they can be classified as distinct species (Roe and Lydeard, 1998).

The lack of such legal muscle may ultimately cause a significant loss in the evolutionary legacy of some species. Clearly then the concept of ESU's and its application to the conservation of cryptic taxa for invertebrates and vertebrates alike requires further study if it is to maintain a strong legal foothold. In an attempt to maximize the conservation of genetic diversity and the evolutionary potential of these five phylogeographic units they should be considered as distinct species to ensure their conservation.

Chapter 7

Congruent patterns of genetic variation in a burrowing freshwater crab revealed by allozymes and mt DNA sequence analysis

Abstract

Five populations of the burrowing freshwater crab, *Potamonautes calcaratus* representing a total of 100 specimens, were collected from the Kruger Nation Park, South Africa. The population genetic structure of this species was investigated using both nuclear genetic markers (allozymes), and direct sequencing of a 610 base pair fragment the cytochrome oxidase I (COI) subunit of the mitochondrial DNA. Electrophoresis of 21 allozyme loci revealed that populations had a moderate degree of genetic differentiation with a $F_{(ST)} = 0.12$. Sequence data for 20 individuals revealed the presence of ten haplotypes, the distribution of which showed no geographic structuring. The Φ_{CT} of 0.43 corroborates a moderate degree of genetic structuring. The nucleotide diversity (π) was low and ranged from 0.00 to 0.007. Sequence divergence amongst populations ranged from 0.49 % to 1.47 %. Both genetic markers revealed moderate population structuring, supporting the conclusion that populations share a common recent ancestry, with moderate levels of recent gene flow. These results provide evidence that allozyme and sequencing data may be congruent and that these independent markers can detect similar patterns of genetic differentiation. Results are discussed in light of contemporary factors that have been likely in sculpting the genetic structure.

Introduction

The genetic structure of populations is largely influenced by the dispersal capacity of the organism and by the availability of suitable habitat. For example, organisms with a high dispersal capacity that live in a continuous environment absent of barriers to gene flow will theoretically show no regional gene frequency divergence. In contrast, aquatic organisms living exclusively in freshwater are often restricted in their distribution, because of the isolating nature of the habitat and a generally poor capacity for dispersal. Aquatic invertebrates, including crustaceans that are restricted to streams and rivers by their lack of effective dispersal mechanisms are often genetically structured when compared to other species with a high dispersal capacity. Examples include the subtropical freshwater atyid shrimps, *Paratya,* and *Caridina* species (Hughes *et al.*, 1995, 1996; Woolschot *et al.*, 1999). Species with a pronounced dispersal capacity, such as freshwater crabs typically exhibit an invariant population genetic structure between drainage systems (Daniels *et al.*, 1998). For example, populations of the mountain stream river crab, *Potamonautes parvispina* (Stewart, 1997b) from two distinct drainage systems in the Western Cape were genetically invariant despite their separation by physical barriers and geographic distance (Daniels *et al.*, 1998a). In addition, similar patterns of genetic homogeneity have been reported for the common Cape river crab, *P. perlatus* (H. Milne-Edwards, 1837) from populations throughout the Western Cape (Daniels *et al.*, 1999a).

Interestingly all the South African freshwater crab species whose population genetic structure has been investigated thus far occur predominantly in lotic systems.

However, two of the freshwater crab species found in South Africa are unique burrow inhabiting animals. One of these species, *Potamonautes calcaratus* (Gordon, 1929) is typically found in burrows around waterholes in the Kruger National Park. Since populations of this species are apparently isolated from large water bodies such as rivers, an opportunity exists to explore the population genetic structure of this species. *P. calcaratus* is expected to be genetically more structured compared to species previous studied (Daniels *et al.*, 1998a, 1999a), considering the habitat requirement of this species.

Allozyme electrophoresis has been widely used to investigate patterns of genetic structure amongst invertebrate populations (Thorpe and Sole Cava, 1994), including decapod crustaceans (Daniels *et al.,* 1998, 1999). This technique has generally revealed low levels of intraspecific variation amongst freshwater crabs (Daniels *et al.*, 1998, 1999). However, few, if any, comparative studies have used more than one genetic marker to detect levels and patterns of genetic differentiation in freshwater crabs. Results from studies on other invertebrates have demonstrated that although genetic markers, such as allozymes and sequencing generally reveal congruence (e.g., Mardulyn *et al.*, 1997; Hurwood and Hughes, 2001; Cox and Hebert, 2001), discordant patterns are just as frequently observed (e.g., Karl and Avise, 1992, Piel and Nutt, 2000). Generally, authors in these latter studies have criticized the use of allozyme electrophoresis for the inference of gene flow, pointing out that allozymes are generally regarded as slowly evolving, and may thus be inappropriate to provide the resolution required.

On the other hand, it could be anticipated that the use of sequencing of a rapidly evolving gene may provide additional insight into the population genetic structure (Piel and Nutt, 2000). Direct sequencing of highly variable mitochondrial genes has proven to be particularly useful to detect genetic variation where allozymes have detected limited variation. The cytochrome oxidase one gene (COI) of the mitochondrial genome has been extensively used in population genetics of invertebrates (Trewick *et al.*, 2000) including crustaceans (Bucklin *et al.*, 1997; Schon *et al.*, 1998; Hurwood and Hughes, 2001), where it has become a useful tool for genetic investigations of population structure. The advantages of using genes from mitochondrial DNA includes the clonal inheritance through the maternal lineage, and the rapid substitution rates of certain regions, making it ideal for population genetic studies. In the present study the population genetic structure of *P. calcaratus* in the Kruger National Park was examined using allozymes and sequencing data from the mitochondrial cytochrome oxidase subunit I to test the hypothesis that populations of freshwater crabs occurring in discontinuous, relatively isolated habitats are genetically more structured, compared to those species that live in lotic environments.

Materials and Methods

Sample collection

Five populations of *Potamonautes calcaratus* were collected from waterholes or pans in the Kruger National Park, South Africa (fig. 7. 1). Crabs were dug from burrows and on capture, were transported alive to the field station and killed by freezing at -20 ^{o}C for 24 h prior to tissue extraction.

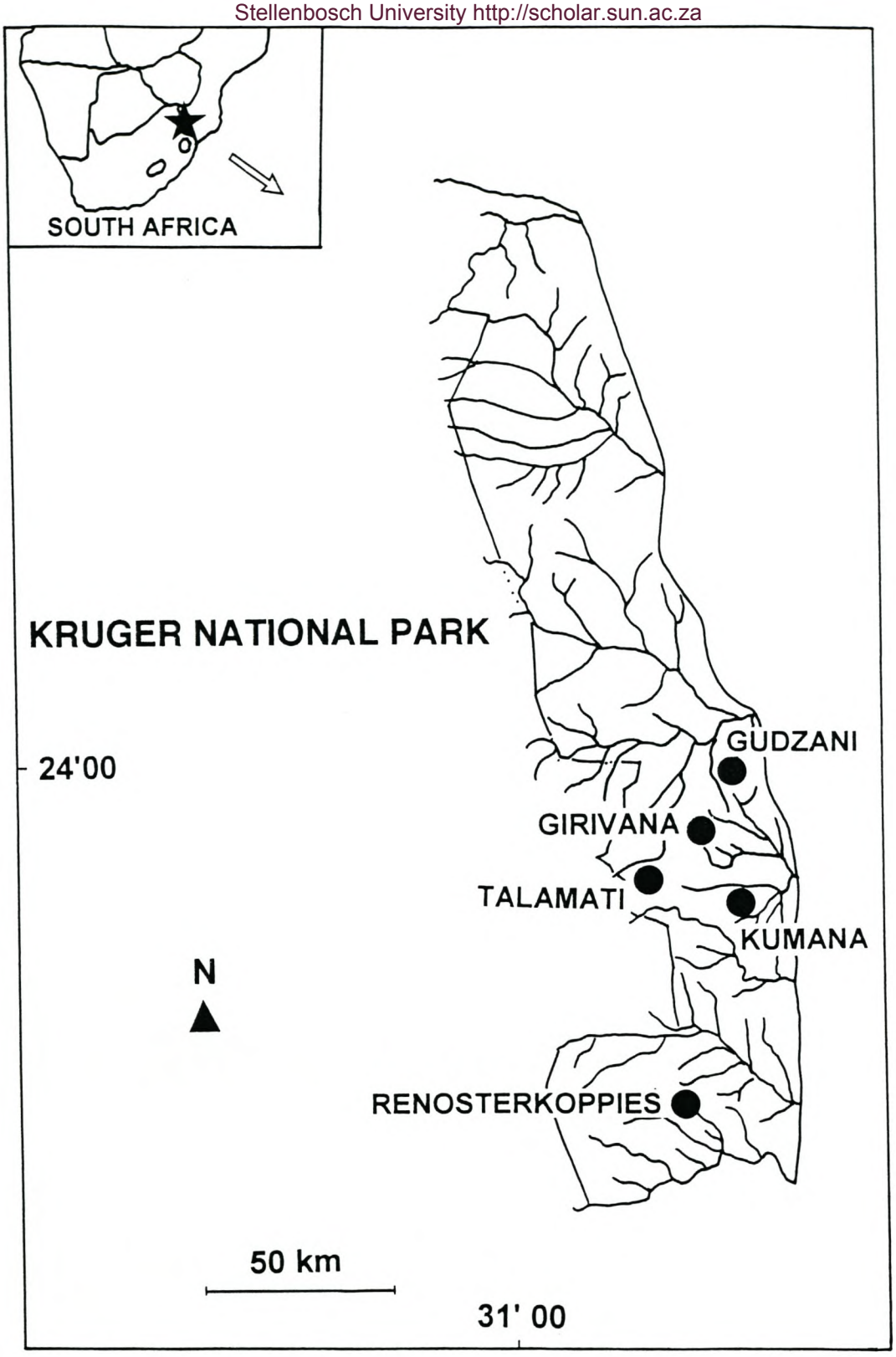


Figure 7. 1. Map showing localities in the Kruger National Park, South Africa.

Allozymes

In addition to the 13 loci examined in chapter 3, five additional loci were examined (table 7. 1). Standard electrophoretic methods were employed as described in chapter 3.

Electrophoretic analyses

The numerical analyses were performed using the BIOSYS-1 programme (Swofford and Selander, 1981). Allelic and genotype frequencies were computed. Chi-square analyses were used to test if populations were in Hardy-Weinberg equilibrium. Levene's (1949) correction for small sample size was used. The mean observed and expected heterozygosity (H_o) and H ($_{exp}$) per locus for each population were calculated using Nei's (1978) unbiased estimates. The mean unbiased genetic identity (*I*) among the populations were calculated from the allelic frequencies according to Nei (1978). The percentage of polymorphic loci in each population was determined. Loci were considered polymorphic if the frequency of the most common allele did not exceed 0.99. The F statistics, including $F_{(IS)}$ (the mean value of genetic differentiation or inbreeding coefficient within subgroups), $F_{(IT)}$ (the mean value of genetic differentiation over the entire population) and $F_{(ST)}$ (the genetic differentiation between any two subpopulations), were calculated to determine the degree of genetic differentiation amongst the populations (Wright, 1965). Values of $F_{(ST)}$ values were tested for significance using the formula given in Waples (1987), $\chi^2 = 2\,N\,F_{(ST)}\,(k - 1)$, where N = the total number of individuals sampled and k = number of alleles at the locus.

Table 7. 1. Enzyme and buffer systems used during electrophoresis. N = the number of loci.

Enzyme	Abbreviation	Buffer	E.C.number	N
Esterase	*Est-1*	C	3.1.1-	1
Glyceraldehyde-3-phosphate	*Gap-1*	C	1.2.1.12	1
Hexokinase	*Hex-1*	C, B	2.7.1.1	1
Peptidase (Leucine glycyl glycyl as substrate)	*Lgg-1*	A	3.14.11-	1
Peptidase (Phenylamide proline as substrate)	*Php-1*	A	3.14.11	1
Superoxide dimutase	*Sod-1*	A, C	1.15.1.1	1

The degrees of freedom are equal to (k - 1) (r -1), where r is the number of populations. An average deviation from random mating expectations was assessed from each population sample by using a multiple-allele, multiple-locus fixation index (Nei, 1977) $F_{(IS)} = H_{exp}-H_{obs}/ H_{exp.}$ Gene flow was estimated using the M method of Slatkin & Voelm (1991), for the overall pairwise comparison between the samples, where M (migration rate, or effective number of migrants exchanged between populations per generation) values were estimated from the mean pairwise $F_{(ST)}$ using the relationship $M= ¼ (1/F_{(ST)} – 1)$.

DNA extraction

Total genomic DNA was isolated from four samples per population. Using the protocol outlined in chapter 6. For the PCR , the primers COI-a and COI-f (Palumbi *et al.*, 1991) were used. PCR clean-up and sequencing procedures were similar to that in chapter 6.

Data Analyses

Each sample was sequenced in both directions and a consensus sequence was created. Sequences were checked for base ambiguity in Sequence Navigator (Applied Biosystems). The COI sequences were aligned by eye only, as no gaps or poorly aligned regions existed within this gene. Sequence data were analyzed using ARLEQUIN 1.1 (Schneider *et al.,* 1997). The degree of population subdivision was estimated using Analysis of Molecular Variance (AMOVA) using the haplotype frequencies. The sequence divergence was calculated using the uncorrected sequence divergence option "p" in PAUP* 4 version 8 beta (Swofford, 2001). In addition, a

minimum spanning network (MSN) was constructed. This is a sensitive method that can be used to reflect the relationships among closely related maternal lineages (Crandall *et al.*, 2000). The absolute number of base changes between the COI sequences was used to calculate the minimum number of substitutions between the different haplotypes. A MSN was constructed manually by connecting the most similar haplotypes by drawing the base pair changes on the branches.

Results

Allozymes

Of the 21 isozymes studied 19 loci (90.47 %) were monomorphic (*Ark-1, Gpi-1, Me-1, Mdh- 2, Idh-1, Idh-2; Lt-1, Ldh-2, Gl-1, Mpi-1, Pgm- 2, Sod-1,Est-1, Lt- 2, Php-1, Lgg-1, Hex-1, Gap-1* and *Pgd-1*). Two loci were polymorphic with the number of alleles ranging from two alleles in *Mdh-1* for the Kumana population to four alleles in *Pgm-1* for all the five populations. The genetic variation at *Pgm-1* was characterized by pronounced heterogeneity. The main allele Pgm^{B} was present in all five populations. Apart from the occurrence of private alleles (Pgm^{D}) in Gudzani, the most northerly population, the remaining four populations all possessed the same two (Pgm^{A}, Pgm^{C}) alleles, at variable frequencies. One of the six (16.66 %) cases of polymorphism observed, was out of Hardy-Weinberg equilibrium. This was due to the deficit of heterozygotes carrying the rare alleles in the Renosterkoppies population at *Pgm-1* ($\chi^2 = 12.788$; $P < 0.05$). Allele frequencies were used to calculate the genetic identities (*I*) between the populations. The genetic *I* obtained for the six populations ranged from 0.992 to 1.000. The percentage of polymorphic loci ranged from 4.8 to 9.5 % and the mean number of alleles per locus was 1.1 (table 7. 2). The mean

observed heterozyosity (H_o) was low and ranged from 0.014 to 0.030.

The pairwise $F_{(ST)}$ amongst samples ranged from 0.017 to 0.127, with a mean of 0.125, indicating a moderate degree of genetic structure. The $F_{(ST)}$ value for the *Mdh-1* locus (0.017) was not statistically significant (χ^2= 3.4). However, the *Pgm-1* locus was statistically significant at 0.127 (χ^2 = 68.58). The overall mean $F_{(ST)}$ of 0.125 was significant (χ^2 = 47.5), and indicates that populations differentiation is moderate, with 12.5 % of the total variation being attributed to differentiation amongst subpopulations. The mean $F_{(ST)}$ value was mainly affected by the allele frequencies differences at the *Pgm-1* locus. The estimate of gene flow (M) based on the overall mean $F_{(ST)}$ was 1.75, suggesting that limited gene exchange is occurring between populations. The $F_{(IS)}$ for locus *Mdh-1* was statistically non significant at –0.022 (χ^2 = -4.40), while the $F_{(IS)}$ for the locus *Pgm-1* was 0.071 and the mean $F_{(IS)}$ of 0.070 with both being statistically significant at χ^2 = 38.34 and χ^2 = 39.9 respectively. The mean $F_{(IT)}$ was at 0.186, indicating the presence of low levels of heterozygosity.

Sequencing

A 660 base pair fragment of the COI gene was amplified. Sequence ambiguity in the first 30 and the last 20 base pairs led to the exclusion of these segments from the data analyses. A total of 610 nucleotides (bp) were used for data analyses. Base compositional bias with unequal proportions of the four bases (A, T, G and C) is common in DNA sequences.

Table 7. 2. The mean number of alleles per locus, the percentage of the loci that were polymorphic and the mean observed heterozygosity (H_o) and the mean expected heterozygosity (H_{exp}) amongst the five populations.

Population	Mean sample size	Mean number of alleles per locus	Percentage of loci polymorphic	H_o	H_{exp}
1. Gudzani	6.0	1.1	4.8	0.016	0.025
2. Girivana	17.0	1.1	4.8	0.018	0.019
3. Kumana	23.0	1.1	9.5	0.030	0.030
4. Talamati	27.9	1.1	4.8	0.026	0.026
5. Renosterkoppies	25.7	1.1	4.8	0.014	0.017

All specimens exhibited a high proportion of A and T (38.73 and 28.11 % average respectively) and lower proportions of C and G respectively (15.58 and 17.56 % average). A total of ten mitochondrial DNA haplotypes was identified, and is presented as a network (fig. 7. 2). Sequences representing all the haplotypes found in this study have been deposited in GenBank (Accession numbers AY042255 – AY042274). The variation within populations ranged from one haplotype in the Renosterkoppies population to four in the Girivana population. Although populations generally shared haplotypes, one haplotype was unique to the Talamati population and two haplotypes were unique to both the Girivana and Kumana populations. From the distribution of haplotypes, it was evident that a number of haplotypes were common and shared among sites, however, no clear geographic grouping was evident amongst haplotypes. The nucleotide diversity (π) within populations was low, and ranged from 0.000 in the Renosterkopppies population to, 0.002 in Gudzani, to 0.004 in the Kumana and Talamati populations with a maximum of 0.007 in the Girivana population. The AMOVA based on haplotype frequencies indicated that among populations variation (Va) was at 43.48 % ($P < 0.05$) while the total variation within populations (Vb) was at 56.52 % ($P < 0.05$). The overall sequence divergence ranged from 0.0 % to 1.47 %.

Discussion

Congruent patterns were observed between the two genetic markers with both allozymes and sequencing revealing moderate levels of genetic variation between populations. Despite the apparently isolated nature of the waterholes, these freshwater

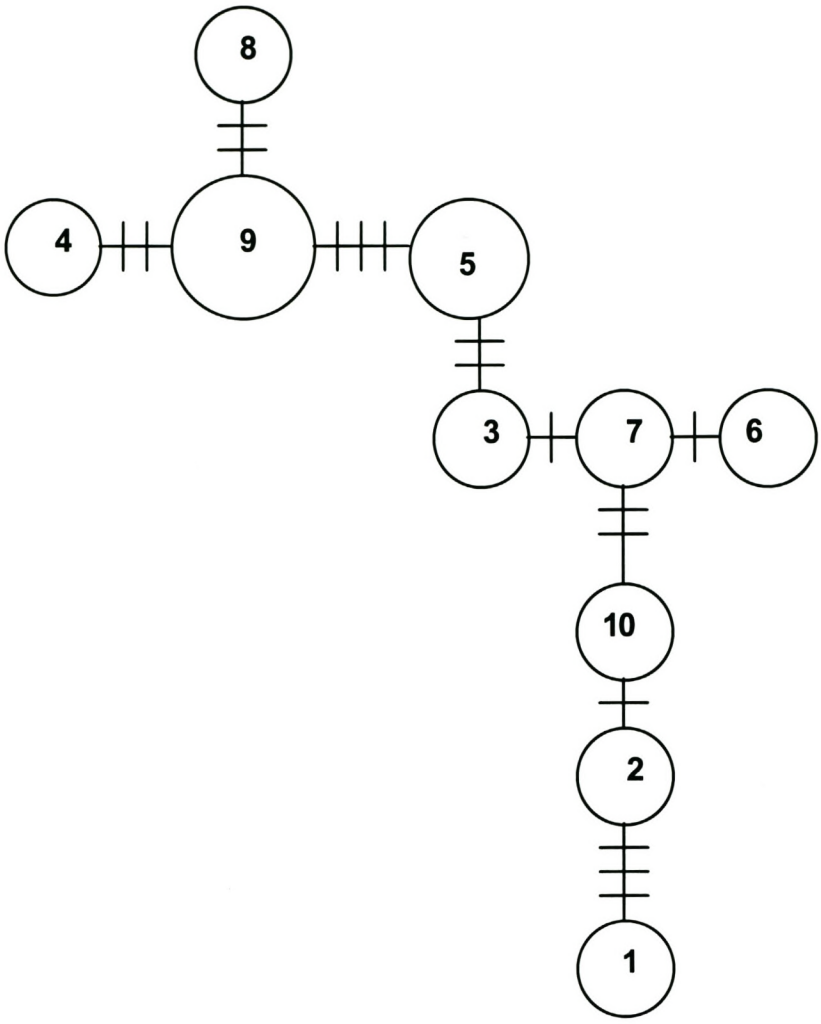


Figure 7. 2. A minimum spanning network of the ten haplotypes.

crabs clearly have a very recent common ancestry, with a moderate level of gene flow. Thus the occurrence of these crabs in apparently isolated habitats does not seem to be a major factor in influencing the population genetic structure of this species. Rather, the contemporary population history coupled with a moderate dispersal capacity is the crucial factors in determining the genetic structure of this freshwater crab species. The COI data clearly indicates that all the animals sampled are probably the result of recent divergence from a common ancestoral haplotype. This argument is favored as no geographic structuring could be detecting with the sequence data among haplotypes between populations that occur in close geographic range. In the absence of geographic structuring among populations, it would be reasonable to speculate that the populations at waterholes are large. Hill and O'Keeffe, (1992) reported that the freshwater crab *Potamonautes perlatus* (H Milne Edwards, 1837) are commonly found in high densities, corroborating the fact that large effective population sizes occur in freshwater crab species. The lack of genetic variation could also be promoted by flooding events that would reduce geographic genetic signatures. The moderate levels of differentiation detected by the mt DNA sequence data in comparison with the allozyme divergence is not unexpected, as mt DNA is expected to differentiate at a faster rate than nuclear DNA because of the maternal inheritance and haploid nature of mt DNA (Birky *et al.,* 1989). The evolutionary scenario that resulted in the present degree of population structure is likely to be the result of a recent colonization event. Gene flow is widespread considering that most haplotypes occurred in more than one population. Dispersal is likely to occur during periods of flooding and by adult crabs. The skewed haplotype frequency observed in the present study is thought to reflect a large population size that allows for the retention of numerous unique haplotypes.

Noticeably, the small sample size in the present study may obscure any significant pattern of variation that could have been detected if a larger sample size was sequenced.

Overall, the dispersal capacity amongst these crabs is probably low, but sufficient for gene flow to override the effects that genetic drift and selection might have on populations. These results indicate that enough gene flow is occurring to swamp any genetic structuring that might have occurred because of the isolated nature of the waterholes. The amphibious nature and dispersal capacity of freshwater crabs is well-documented (Barbaresi *et al.*, 1997). Considering that *P. calcaratus* lives in waterholes, and is the most terrestrial freshwater crab investigated thus far. This species may thus be well adapted for dispersal over land. More recently, Hurwood and Hughes (2001), using allozymes and sequencing of COI found that the freshwater shrimp, *Caridina zebra* is capable of terrestrial dispersal.

The low genetic variability detected using allozymes in this species has also been recorded in other intraspecific studies on freshwater crab species (Daniels *et al.*, 1998, 1999). For example populations of two mountain stream species, *P. parvispina* and *P. clarus* (Gouws *et al.*, 2000) had a mean $F_{(ST)}$ values of 0.032 and 0.085 respectfully, (Daniels *et al.*, 1998; unpublished data) despite the fact that some of the populations of these species were collected from different drainage systems separated by on average by 25 km of mountainous terrain. Such data would argue for high dispersal despite considerable physical barriers. In contrast to this, the swamp forest species, *P. lividus* (Gouws *et al.*, 2001) had a mean $F_{(ST)}$ value of 0.314. The mean $F_{(ST)}$ value of

0.125 reported for *P. calcaratus* is greater than that reported for mountain stream species and more similar to that reported in *P. lividus*. Interestingly the latter two species are the among the most terrestrial freshwater crab species found in South Africa. The high mean $F_{(ST)}$ reported for *P. lividus* is not surprising as the swamp forests occur as isolated habitat separated by 35 km, while *P. calcaratus* populations were on average separated by 25km. The occurrence of these isolated habitats thus appear to have a moderate effect on genetic structure as evident from the varying levels of genetic differentiation derived from the $F_{(ST)}$ data for the different habitat types. Generally, it appears that the burrowing crab species exhibit higher levels if genetic differentiation when compared to mountain stream animals.

The genetic structure between populations of *P. calcaratus* lend marginal support to the hypothesis that population structure is directly related to habitat structure. In addition it would appear that the contemporary population history and dispersal capacity of an organism are curtail in determining the genetic structure of a population. Hebert *et al.*, (2000) recently demonstrated that attempts to predict population genetic structure from their habitat are limited. Their study revealed that habitat structure analysis constituted an important but insufficient predictor of genetic structure, indicating that historical demography and factors that affect genetic diversity (gene flow, drift and selection, and population history) should be investigated, in an attempt to explain the level of genetic variation observed. A general conclusion that can be drawn from this is that levels of conspecific genetic variation as detected using allozyme data is low amongst freshwater crabs despite apparent habitat isolation and that this is likely to be due to recent separation (or range

expansion) of populations and moderate dispersal levels.

Langor and Sperling (1997) reported that in general, less than 2 % sequence divergence of variation is evident among conspecific invertebrate populations using COI. Similar low levels of variation were observed in the present study. The low maximum sequence divergence of 1.47 % among populations of *Potamonautes calcaratus*, is similar to intraspecific levels of genetic variation detected in invertebrates, including other crustaceans. For example, unpublished COI sequence data (Daniels unpublished data) from multiple representatives from three freshwater crab species *P. perlatus* (H Milne -Edwards, 1837) *P. unispinus* (Stewart and Cook, 1998) and *P sidneyi* (Rathbun, 1904) revealed that conspecific populations generally have sequence divergence values in the region of 2 %. Using a similar fragment of the COI gene, Barber and Erdmann (2000) reported a 1.4 % variation between stomatopod populations of *Gonodactylaceus mutatus* (Lanchester, 1903) from Indonesea and Hawaii. Schon *et al.*, (1998) reported intraspecific variation of 2.1 % amongst European populations of the non-marine ostracod, *Darwinula stevensoni*, while Perez *et al.*, (1994) reported values as high as 3.8 % for *Artemia*. Edmands (1999) showed that sequence divergence for the intertidal copepod *Tigriopus californicus* examined on a micro and meso-spatial scales, may vary from as low as 0.2 % to as high as 22.3 %. This author demonstrated that sequence divergence was highly correlated with geographic distance. All the above studies are based on COI sequence data, and underline the utility of this gene in detecting population genetic structure.

Nucleotide diversity (π) is commonly used to measures differences between haplotypes. Stephan and Langley (1992) reported that for most species, this value ranges between 0.0005 and 0.020. The low mean nucleotide diversity (π) of 0.0039 recorded in the present study was similar to that of 0.0042 for the marine copepod *Calanus finmarchinus* (Bucklin and Kocher, 1996) and nearly identical to the 0.0038 value reported for the euphausiid, *Meganyctiphanes norvegica* (M. Sars) (Bucklin *et al.*, 1997). More recently, Gomez-Zurita *et al.*, (2000) reported a nucleotide diversity of 0.004 for the leaf beetle, *Timarcha geottingensis*. It would appear that nucleotide diversity in populations of invertebrate species is generally low.

Chapter 8

Phylogenetic relationships of the southern African freshwater crab fauna (Decapoda: Potamonautidae: *Potamonautes*) derived from multiple data

Abstract

The phylogenetic relationships among the southern African freshwater crab species were examined using partial sequence data from 12 S rRNA and 16 S rRNA mtDNA genes, morphology and allozymes. The aims were firstly to determine whether freshwater crabs that live in the same river systems share a close phylogenetic relationship. Secondly, to investigate whether hybridizing taxa are closely related and thirdly, to quantify the subgeneric divisions proposed by Bott (1955). Phylogenetic analysis based on sequence data revealed largely congruent tree topologies supported with moderate to high bootstrap values. The morphological data failed to recover distinct clades, while the allozyme data generally supported patterns recovered by the sequence data. A combined analysis of all three data sets suggested that mountain stream species (*P. depressus*, *P. clarus*, *P. parvispina*, *P. brincki* and *P. parvicorpus*) are polyphyletic, while large bodied riverine species form a monophyletic group. Clear biogeographic patterning could be detected for the large bodied riverine species. In addition, there was a clear correlation between genetic distance values and the ability of sympatric species to hybridize. Our results suggest recent rapid cladogenesis, marked in most instances by stasis in morphological characters. Known hybrid pairs were closely related. The results did not support previously proposed subgeneric designations.

Introduction

Freshwater crabs are distributed throughout tropical, subtropical and temperate regions of Central and South America, Africa, Madagascar, southern Europe, Asia and Australia. Members of the group inhabit a variety of ecological niches ranging from high mountain streams, rivers, lakes, swamps, forests and dry savannahs (e.g. Von Sternberg *et al.*, 1999a). Globally, the higher level taxonomic relationships among freshwater crab families has been unstable and the number of extant recognised families varies between eight and 11 (Bott, 1970; Cumberlidge 1999a). Following the taxonomic designations suggested by Cumberlidge (1999a), at least four freshwater crab families (Potamidae; Potamonautidae; Deckeniidae and Platythelphusidae) and a single subfamily (Globonautinae), occur throughout Africa.

Pertinent to the focus of this study, the African family Potamonautidae contains four endemic genera, *Liberonautes*, *Potamonautes, Potamonemus* and *Sudanonautes* (Cumberlidge, 1999a). *Potamonautes* is the most widely distributed and species rich, occurring from the sub-Saharan region southwards to South Africa. Initially, *Potamonautes* was thought to be comprised 15 subgenera and 38 species (Bott, 1955). Alpha-taxonomic studies of this group have however led to the description of several new species, and the genus currently comprises in excess of 60 species (Cumberlidge, 1999a). Recent systematic endeavours on the South African *Potamonautes* alone has led to the discovery of seven new species (e.g. Stewart, 1997a; Stewart and Cook, 1998, Daniels *et al.,* 1998, 2001b; Gouws *et al.*, 2000; 2001).

The development of modern systematic methodology has greatly advanced our understanding of the patterns of diversification of aquatic organisms and, moreover, also provided valuable information for phylogenetic analysis (Avise, 1994). Recent empirical work on freshwater crustaceans suggests that their confinement to freshwater systems render them closely linked to the dynamics of geological and hydrographic history of an area (Taylor *et al.*, 1996, 1998). However, the use of freshwater crabs as biogeographic indicators has been controversial (Banarescu, 1990; Ng and Rodriguez, 1995b). Banarescu (1990) suggested that freshwater crabs are poor indicators for biogeographic studies when compared to freshwater crayfishes. Freshwater crabs can survive outside water and disperse short distances over land making them less restricted to their drainage systems. Ng and Rodriguez (1995) strongly dispute Banarescu's views based on previous morphological cladistic analysis of South American freshwater crabs (Rodriguez and Campos, 1989; Rodriguez and Pereira, 1992b) and maintain that freshwater crabs are invaluable indicators for zoogeographic studies.

Freshwater crabs have a moderate dispersal capacity, and investigations into the fauna of South Africa (and globally) have demonstrated that most species have specific habitat requirements and narrow distribution ranges (Ng and Rodriguez, 1995). The Southern African freshwater crab species are characterized by interesting distribution patterns. Most river systems contain at least two freshwater crab species, a mountain stream and mid to lower riverine species, and these taxa sometimes overlap in a narrow zone of sympatry (Daniels *et al.*, 1998, Gouws *et al.*, 2000). Bott (1955) speculated that the freshwater crabs in southern Africa are derived from species that

migrated southwards through river systems from central Africa. Geological and hydrological evidence suggest that historically, southern African rivers were connected to those from Central and North Africa (Skelton, 1994). In addition, temporal and spatial isolation among populations and drainages could have caused allopatric divergence among taxa, leading to speciation within the widespread ancestral form (also see Hewitt, 1996, 2000). On the other hand, biological factors such as competition and selection could also promote radiation and thus lead to taxa occupying different ecological niches within the same river system. If the latter arguments hold, species occurring in the same river catchment should be genetically more closely related to each other than to species in geographically more distant systems. An examination of phylogenetic relationships among the southern African freshwater crab species could shed light into which factors have sculpted the contemporary distribution of species.

Pronounced levels of variability in diagnostic morphological features had led earlier researchers to conclude that freshwater crab species hybridize frequently (Barnard, 1935, 1950). Allozyme electrophoresis has revealed the existence of four hybridising species pairs among the South African freshwater crab fauna: namely, *P. perlatus* and *P. granularis*; *P. perlatus* and *P. sidneyi*; *P. sidneyi* and *P. unispinus;* and *P. depressus* and *P. clarus*. In addition, mitochondrial DNA sequencing has revealed introgression between and *P. depressus* and *P. clarus* (chapter 6). Not all species pairs that occur sympatrically hybridize and it remains unclear whether hybridizing freshwater crab species are genetically closely related.

At the higher taxonomic level (for example between different families) it would appear that the likelihood of hybridization is proportional to genetic similarity (Smith, 1992). At the species level however, the amount of divergence might not be a constraint on the capacity of taxa to hybridize (Smith, 1992). Smith (1992) is of the opinion that reproductive compatibility among species may or may not be blocked by subsequent evolution and the potential for gene exchange might thus not be indicative of a close phylogenetic relationship. The position of hybridizing taxa in phylogenetics is troublesome because it can distort phylogenetic relationships, and it remains unclear whether the potential for reproductive compatibility (hybridisation) is indicative of a close phylogenetic relationship, or if the potential for gene exchange is only loosely coupled to relatedness (McDade, 1990, 1992; Sang and Zhong, 2000).

Despite Bott's (1955) taxonomic studies, *Potamonautes* has remained recalcitrant to the tools of modern systematics and the validity of these subgeneric divisions remain speculative (Cumberlidge, 1999a). Bott (1955) argues for the recognition of fifteen subgenera based on morphological characters in *Potamonautes* of which three subgenera occur in southern African. They include the subgenus *Potamonautes* comprising *P. warreni, P. bayonianus, P. dubius* and *P. perlatus*; the subgenus *Obesopotamonautes* contains *P. langi*, *P. obesus* and *P. calcaratus;* and the subgenus *Orthopotamonautes* includes *P. depressus, P. dybowskii* and *P. sidneyi*. The species *P. obesus*, *P. langi* and *P. dybowskii* are extralimital to the region.

By making use of data derived from two mitochondrial genes (12 S rRNA and 16 S rRNA mt DNA), allozymes and morphological characters the following objectives are

addressed: Firstly, to investigate whether freshwater crab species living in the same river systems share a close phylogenetic relationship. Secondly, to determine whether hybridizing taxa are genetically closely related and thirdly, to examine the validity of the subgeneric divisions proposed by Bott (1955).

Materials and methods

Sample collection

Southern Africa is defined as the area south of the Kunene and the Zambezi rivers (Werger, 1978) and includes Namibia, Botswana, Zimbabwe, Mozambique and South Africa (fig. 8. 1). These two northern rivers constitute the natural northern boundary of the sub-continent (Skelton, 1994).

With the exception of *Potamonautes bayonianus* (Okovango Delta, Botswana) which was hand caught and stored in 95 % ethanol, all other species were collected using baited lines and killed by refrigeration at –20 ^{0}C, followed by muscle tissue dissection. These tissue samples were subsequently preserved in liquid nitrogen in the field and later transferred to nunc (cryogenic) tubes and stored at either –20 or –80 ^{0}C until required for allozyme electrophoresis or DNA extraction.

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from a single representative of each of the 14 southern African *Potamonautes* species (table 8. 1). Prior to DNA extraction, tissue samples were washed in sterile water. Total genomic DNA was isolated from 0.5 to 1 g of muscle tissue. Samples were digested in sterilized eppendorfs containing 500 µl

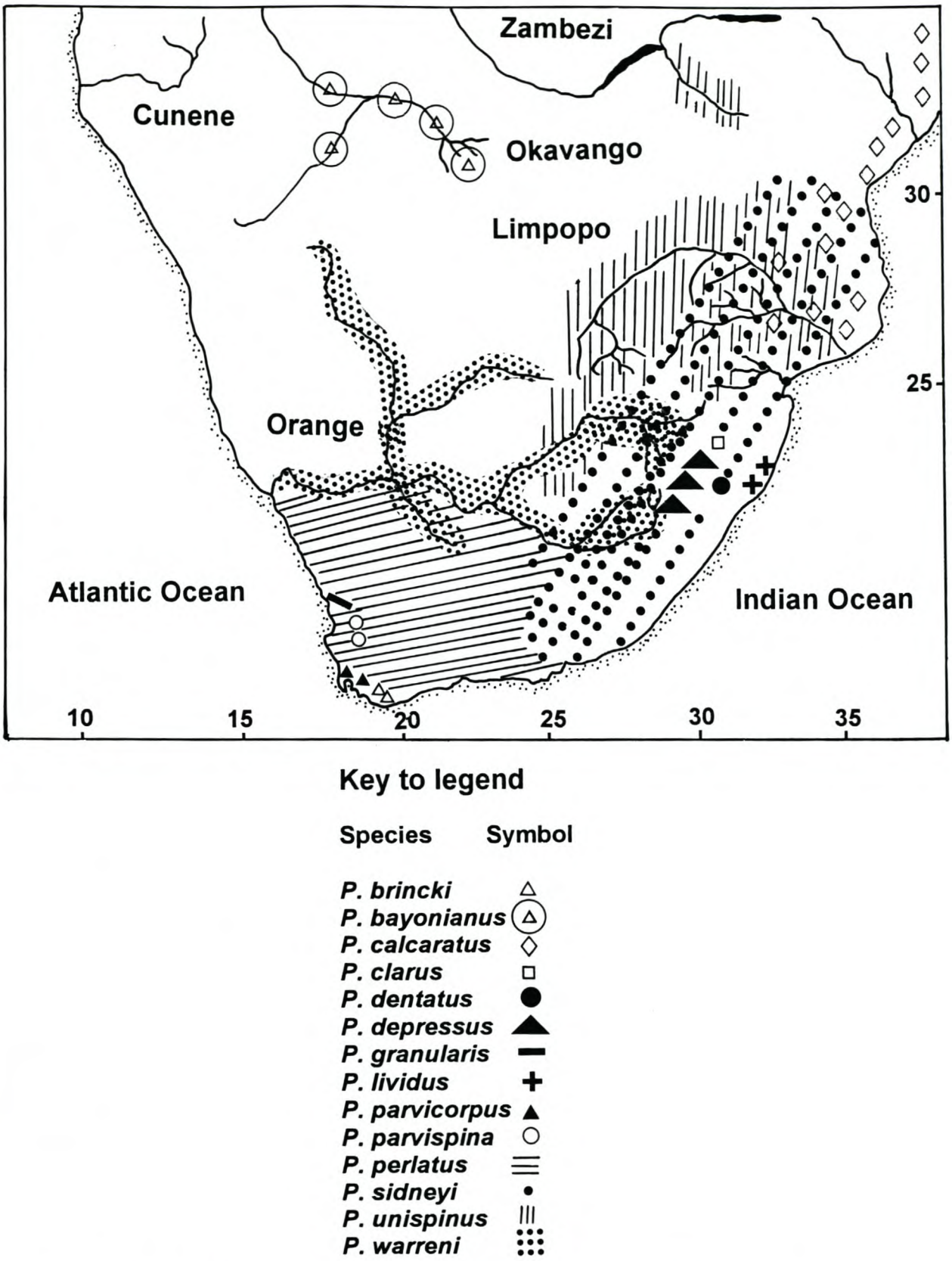


Figure 8. 1. A map showing the major drainage systems in southern Africa.

Table 8. 1. List of the 14 freshwater crab species studied, collection locality and main habitat type.

Species	Collection locality	Main habitat type
P. bayonianus	Okavango Delta, Botswana	riverine
P. brincki	Fernkloof, Western Cape	mountain streams
P. calcaratus	Renosterkoppies, Northern Province	waterholes
P. clarus	Oliviershoekpass, KwaZulu-Natal	mountain streams
P. dentatus	Mgeni River, KwaZulu-Natal	riverine
P. depressus	Coleford, KwaZulu-Natal	mountain streams
P. granularis	Lutzville, Western Cape	riverine
P. lividus	Empangeni, KwaZulu-Natal	swamp forests
P. parvicorpus	Blinkwater, Western Cape	mountain streams
P. parvispina	Algeria, Western Cape	mountain streams
P. perlatus	Citrusdal, Western Cape	riverine
P. sidneyi	Empangeni, KwaZulu-Natal	riverine
P. unispinus	Shingwedzi, Northern Province	riverine
P. warreni	Bothaville, Free State	riverine

of DNA lysis buffer, 20 µl of proteinase K at a concentration of 10 mg/ml and 10 µl of RNAse at a similar concentration. This mixture was incubated for one hour at 50 ^{0}C. DNA was extracted using the phenol/chloroform: isoamylalcohol method outlined in Hillis *et al.,* (1996). DNA concentrations were determined spectrophotometrically and samples diluted to a final concentration of 40 ng/µl. DNA samples were stored at –20 ^{0}C until needed.

Two mitochondrial genes, 16 S rRNA and 12 S rRNA were selected for the present study as they have been successfully used in evolutionary studies in a wide range of decapod crustaceans (e.g. Cunningham *et al.*, 1992; Strumbauer *et al.*, 1996; Schubart *et al.*, 1998). The primers 16 Sar and 16 Sbr (Cunningham *et al.*, 1992) were used to amplify the 16 S rRNA gene, while the primers 12 Sai and 12Smb (Kocher *et al.*, 1989) were used to amplify the12 S rRNA gene. For each PCR a 25 µl reaction was performed that contained 14.9 µl of millipore water, 3µl of a 25 µm $MgCl_2$, 2.5 µl of 10 x Mg^{2+} free buffer, 0.5 µl of a 10mM dNTP solution and 0.5 µl of each primer set (at 10 µM each), 0.1Unit of Taq polymerase and 3 µl of template DNA. The PCR temperature regime for both gene fragments was 95 ^{0}C for 2 min; 95 ^{0}C for 30 s; 50 ^{0}C for 40 s; 72 ^{0}C for 1min and then 34 cycles for the last three steps, followed by a final extension of 10 min at 72 ^{0}C. PCR products were electrophoresed in a 1 % regular agarose gel containing ethidium bromide for 30 min at 70 V. Products were visualized under UV light. PCR products were purified using a PCR purification kit (Qiagen). Purified PCR products were cycle sequenced following the recommendations of the manufacturer (Applied Biosystems). Unincorporated dideoxynucleotides were removed by gel filtration using Sephadex G-25 (Sigma).

Sequencing was performed on an ABI 377 automated machine.

Phylogenetic analysis

To improve accuracy both heavy and light strands were sequenced and a consensus sequence was created for each taxon. Sequences were scrutinized for base ambiguity in Sequence Navigator (Applied Biosystems). The 16 S r RNA and 12 S rRNA sequence data were aligned in CLUSTAL W version 1.6 (Thompson *et al.*, 1994) using the default parameters and sequences were further adjusted by eye to optimize the alignment. Ambiguous regions that could not be aligned with certainty were excluded from the phylogenetic analysis. The 12 S r RNA and 16 S r RNA sequences from this study have been deposited in GenBank (12 S rRNA accession numbers AY042241 – AY 042252; 16 S rRNA accession numbers AY042275 - AY042319).

The selection of an appropriate outgroup for phylogenetic analysis was problematic due to the uncertain sister taxon relationships of *Potamonautes* (Cumberlidge, 1999a). Preliminary sequence data for *Potamon fluviatilus* and *Sudanonautes floweri* indicated that *Potamonautes* is equidistant from both genera. Analysis of the sequence data including the two outgroups consistently placed *P. calcaratus* basal in the monophyletic *Potamonautes* clade. The latter species was defined as a single outgroup to reduce the addition of noise when distantly related outgroups are used (Halanych and Robinson, 1999).

Phylogenetic data analyses were executed in PAUP*4 version beta 8 (Swofford, 2001) using maximum parsimony (MP), neighbor joining (NJ) and maximum likelihood

(ML) methods. For the MP analysis trees were generated using the heuristic search option with TBR branch swapping and a 1000 random taxon additions. Furthermore, for the MP analysis both unweighted and weighted analysis (transversions weighted at 2:1 over transitions) were performed. Recently, ML analysis has become particularly favored as it permits the application of mathematical models resulting in a more powerful inference (Whelan *et al.*, 2001; Posada and Crandall, 2001). For the ML analysis the appropriate substitution model was calculated using MODELTEST version 3. 06 (Posada and Crandall, 1998). The optimal ML model for the sequence data sets were incorporated in the calculation of sequence divergence values from which NJ topologies were constructed. Confidence in the nodes recovered by MP and NJ was estimated by performing 1000 bootstrap replicates (Felsenstein, 1985). Due to time constraints only 100 replicates were performed for ML. In this study, we regard bootstrap values < 50 % as nodes not supported, bootstrap values between 50 and 70 % as weakly supported and values >70 % as supported.

In addition, the data sets were combined after performing the Partition-homogeneity tests / IDL (Farris *et al.*, 1995), as implemented in PAUP. All tests were run with both uninformative characters excluded (Cunningham, 1997) and included. More recently, Lee (2001) demonstrated that unless all uninformative characters (invariant and autapomorphic) are excluded, the partition homogeneity test may overestimate the amount of incongruence, especially when morphological and molecular data sets are compared (Yoder *et al.*, 2001). Irrespective of the validity of the ILD test, the sequence data were combined as both genes are in effect linked on the mitochondrial genome.

The relationship between sympatric hybridizing and non hybridizing sympatric species pairs was examined by calculating the sequence divergence among each species pair. The mean sequence divergence values for each of the two genes were compared using a students *t* - test in the statistical package STATISTICA (StatSoftInc., 1996). The Shimodaira-Hasegawa (Shimodaira-Hasegawa, 1999) test with full optimization and 1000 bootstrap replicates as implemented in PAUP*4 version beta 8 (Swofford, 2001) were used to test alternative hypotheses. Topological constraints were specified using MacClade (Maddison and Maddison, 1992).

Comparative morphology

Adult specimens of each taxon housed in the South African Museum collection were examined to assess the degree of morphological variation exhibited by characters. In addition, morphometric characters derived from published (Stewart, 1997; Stewart and Cook, 1998, Daniels *et al.,* 1998; Gouws *et al.*, 2000; 2001; Daniels *et al.*, 2001) and unpublished data (Daniels, Stewart and Gouws unpublished) were used in the morphological phylogeny. A total of 26 morphological characters derived from carapace, mandibular palp, pereiopods and pleopods (gonopod 1) were scored for phylogenetic analysis (Appendix 4). These characters were chosen based on their taxonomic utility in diagnosing *Potamonautes* species (see Cumberlidge, 1999a). Autapomorphic characters were excluded from the analysis. A detailed discussion of the usefulness of the morphological characters used are presented in Appendix 5. A complete data matrix is given in table 8. 2. The morphological data were analysed using parsimony method as implemented in PAUP*4 version beta 8 (Swofford, 2001) based on ordered characters with equal weighting. Successive weighting of characters

was conducted in an attempt to improve the resolution. The topology of the wieghted tree was compared with the topology obtained with equal weights. The robustness of nodes were assessed using bootstrap replicates (Felsenstein, 1985).

Allozyme analysis

Genetic variation among conspecific freshwater crab populations is low (e.g. Daniels *et al.,* 1999, 2001). Three populations for each species were randomly selected and pooled and species used as terminal points to represent each of the 13 freshwater crab species, excluding *P. bayonianus,* for which no allozyme data were available. Data for this species were coded as missing in the combined analysis. These populations were subjected to allozyme electrophoresis on three buffer systems and stained for the appropriate locus following the general staining protocol outlined in earlier studies on the freshwater crabs (Daniels *et al.*, 1999; 2001). A total of 10 putative loci encoding 14 enzyme loci were examined (table 8. 3).The appropriate analytical methods for allozyme electrophoresis in phylogenetics have remained a contentious issue (Wiens, 1995; Swofford *et al.*, 1996; Murphy and Doyle, 1998). More recently, Wiens (2000a) demonstrated that allozyme data could recover congruent phylogenetic relationships similar to that derived from sequence data and morphology when the allozyme data are analysed using distance and continuous maximum likelihood methods.

Table 8. 2. Character matrix of the 26 morphological characters for the 14 potamonautid species.

Species	Characters																									
P. brincki	2	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	2	1	1	2	1	0	0	0	1
P. bayonianus	0	1	0	1	0	1	0	1	1	1	1	1	1	0	1	1	1	2	1	0	2	0	0	0	0	1
P. calcaratus	1	1	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	2	1	1	0	1	0	1	0	0
P. clarus	2	1	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	2	0	1	0	1	1	1	1	1
P. dentatus	0	0	0	1	0	0	0	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0	0	1	1
P. depressus	2	1	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0
P. granularis	2	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	0	1	1	1	0	1	0	0	0
P. lividus	2	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	2	0	2	2	1	0	1	0	0
P. parvicorpus	2	1	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	2	1	1	1	1	0	0	0	0
P. parvispina	0	1	0	1	0	1	1	1	1	1	1	1	1	0	1	1	1	2	1	1	2	1	0	1	0	1
P. perlatus	2	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	2	1	0	0	0	0
P. sidneyi	2	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	2	0	0	0	0	0
P. unispinus	0	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	0	1	1	1	0	1	0	0	0
P. warreni	0	0	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	0	1	1	1	0	1	1	0	0

Table 8. 3. Enzyme and buffer systems used during electrophoresis. N = the number of loci.

Enzyme	Abbreviation	Buffer	E.C.number	N
Arginine kinase	*Ark-1*	A	2.7.3.3	1
Glucose phosphate isomerase	*Gpi-1*	A	5.3.1.9	1
Isocitric dehydrogenase	*Idh-1, 2*	B	1.1.1.42	2
Lactate dehydrogenase	*Ldh-1*	B	1.1.1.27	1
Peptidase (Glycyl leucine as substrate)	*Gl-1*	B	3.4.11-	1
Peptidase (Leucine tyrosine as substrate)	*Lt-1, 2*	A	3.4.11-	2
Malate dehydrogenase	*Mdh-1, 2*	C	1.1.1.37	2
Malic enzyme	*Me-1*	C	1.1.1.40	1
Mannose-phosphate isomerase	*Mpi-1*	B	5.3.1.8	1
Phosphoglucomutase	*Pgm-1, 2*	A	2.7.5.1	2

Wiens (2000a) suggested that, among the distance based methods, the choice of distance measures has relatively little impact on the overall performance of the method. However, he concluded that the Rogers (1972) and modified Roger's (Wright, 1978) distance had slightly higher accuracies when compared to Prevosti's (Wright, 1978) and unbiased Nei's distances (Nei, 1978). These four distance methods outlined above were implemented in BIOSYS-1 (Swofford and Selander, 1981) using the UPGMA tree reconstruction method. For the four distance methods, the trees were unrooted. In addition NJ method (neighbour-joining, using uncorrected sequence distances) analyses were performed in PAUP*4 version beta 8 (Swofford, 2001).

Combined analysis

The analysis of multiple data sets in phylogenetics has been the subject of debate (Heulsenbeck *et al.*, 1997; Cunningham, 1997). Two schools of thought exist, taxonomic congruence and total evidence. The taxonomic congruence approach argues for the independent analysis of data sets (Miyamotao and Fitch, 1995). The total evidence approach argues that all the data should be combined into a single data set for analysis (Kluge, 1989). In the present study we employ both methods, by first analysing data sets independently and then combining data sets. The partition homogeneity test (Farris *et al.*, 1995) was once again used to evaluate the combinality of the different data partitions. Data partitions were as follow, 12 S rRNA - allozymes, 12 S rRNA - morphology, 16 S rRNA - allozymes, 16 S rRNA - morphology, combined DNA data - allozymes, DNA data - morphology, morphology – allozymes and total evidence (12 S rRNA, 16 S rRNA, allozymes and morphology).

These tests were conducted in PAUP*4 version beta 8 (Swofford, 2001), with 1000 partition homogeneity replicates. The overall performance of the ILD tests (where uninformative characters were included or excluded) were explored by using a students *t* - test in the statistical package STATISTICA (StatSoftInc., 1996). For the allozyme data, loci were coded as characters according to the *minimum allele turnover model* (Table 8. 4) of Mickevich and Mitter (1983). Data were analysed using MP in PAUP *4 version beta 8 (Swofford, 2001).

Results

Characteristics of the ribosomal DNA data

A total of 324 and 479 base pairs were sequenced for 12 S rRNA and 16 S rRNA genes respectively. For the 12 S rRNA data, the maximum likelihood model selected the model HKY 85 (Hasegawa *et al.,* 1985) + Γ. This model incorporates different base frequencies. In addition the transition to transversion ratio of 3.9988 was calculated for this gene. There was also significant rate heterogeneity in the data, this is expressed using gamma distribution with the shape parameter (α). The estimated shape parameter for the gamma distribution of the data, was $\alpha = 0.1874$. Incorporating this model of molecular evolution, the phylogenetic relationships among taxa were estimated using maximum likelihood (-1n L = 1090.633, fig. 8. 2). The sister taxon relationships between *P. clarus* and *P. depressus*; *P brincki* and *P. parvicorpus*; and *P. granularis* and *P. perlatus* are well supported.

Table 8. 4. Character matrix used in phylogenetic analysis of the 13 potamonatid species (excluding *P. bayonianus* as no allozyme data are available for this species) studied. Loci are coded as characters, under the *minimum allele turnover model* of Mickevich and Mitter (1983).

	Characters
Species	1 2 3 4 5 6 7 8 9 10 11 12 13 14
P. brincki	1 2 5 2 0 0 2 5 1 1 4 0 2 1
P. calcaratus	0 0 0 0 0 0 0 0 0 0 0 0 0 0
P. clarus	2 4 1 2 0 3 3 4 1 3 3 1 2 1
P. dentatus	0 3 4 0 0 0 2 1 1 5 4 0 0 3
P. depressus	0 0 1 1 0 2 1 1 1 1 5 2 2 1
P. granularis	0 1 6 2 0 0 2 4 1 6 1 0 2 2
P. lividus	0 5 2 0 0 0 0 2 1 6 1 0 2 2
P. parvicorpus	1 2 2 0 0 0 2 5 1 3 2 0 2 1
P. parvispina	0 2 4 2 0 1 2 5 1 3 6 0 2 1
P. perlatus	0 1 2 2 0 0 2 4 1 3 1 0 2 2
P. sidneyi	0 1 3 0 0 0 4 1 1 2 1 0 0 2
P. unispinus	0 3 2 0 0 0 2 3 1 4 4 0 0 1
P. warreni	0 3 2 0 0 0 2 1 1 4 4 0 1 1

Characters: (1) *Ark-1*, (2) *Idh-2*, (3) *Gpi-1*, (4) *Me -1*, (5) *Mdh-1*, (6) *Mdh-2*, (7) *Idh-1*, (8) *Lt-1*, (9) *Ldh-1*, (10) *Pgm-1*, (11) *Gl-1*, (12) *Mpi-1*, (13) *Pgm-2* and (14) *Lt-2*.

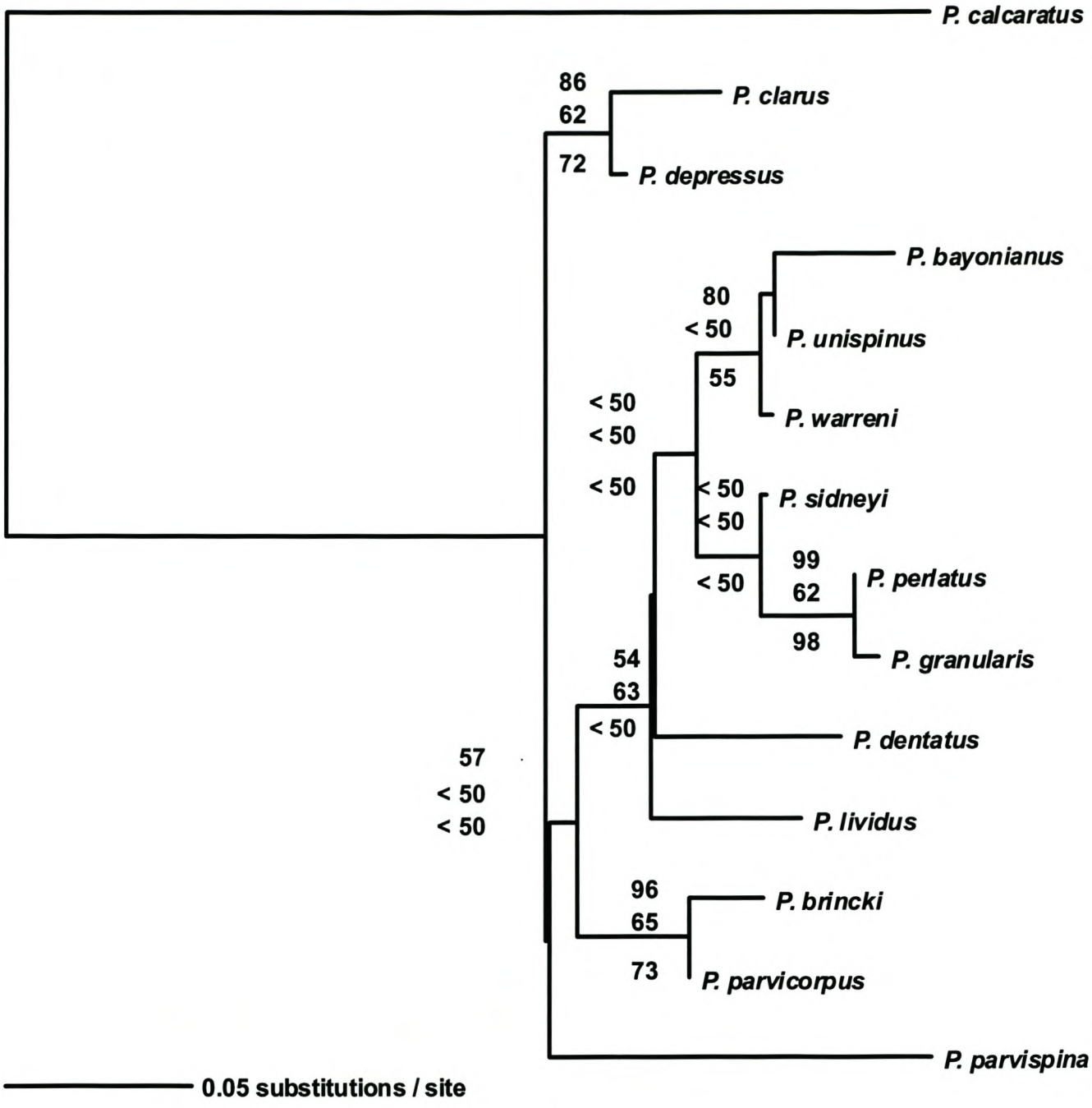


Figure 8. 2. The ML for the 12 S rRNA mt gene for the 14 southern African river crab species. Bootstrap value > 50% are shown on the branches. Values above the branch are for MP and NJ, while the value below is for ML. Branch lengths are shown proportional to the amount of change along the branch.

The MP analysis recovered a single tree (table 8. 5). The relationships between the riverine crabs with carapace dentition, *P. warreni*, *P. unispinus* and *P. bayonianus* are well supported (80 % bootstrap). Furthermore the topology of the MP tree was identical to ML.

16 S sequence topology

The GRT (Rodriguez *et al.*, 1990) + Γ model was used for ML. This model incorporates differences in base frequencies, unequal transition and transversion rates and a different estimated rate was used for each of the six variables (R_1= R_3= R_4 = R_6 = 1.00; R_2 = 6.10; R_5 = 2.79). The estimated shape parameter for α was 0.3484. Incorporating this model of molecular evolution, a phylogenetic relationship was estimated using maximum likelihood (-1n L = 1969.6141, fig 8.3). Congruent with the 12 S topology presented above, the 16 S topology recovered the relationships *P. depressus* and *P. clarus*, *P. warreni* and *P. unispinus*, *P. perlatus* and *P. granularis*. In contrast, the 16 S topology strongly supports the monophyly of the large bodied riverine freshwater crabs with 91 % bootstrap support. In addition, the 16 S topology clearly placed *P. dentatus* and *P. lividus* as sister taxa to the large bodied riverine species (72% support). The NJ topology lends marginal support to the relationships between the large bodied crab species. The MP analysis recovered a single tree (table 8. 5) that supported the same relationships derived from ML.

Table 8. 5. Breakdown of the statistics derived from each gene used in this study. Parsimony informative characters (P.I.C). N bases is the number of base pairs sequenced.

Gene	N bases	Base Frequencies				P.I. C.	CI	RI	Tree length
		A	C	G	T				
12 S rRNA	324	34.73	10.33	18.01	36.91	30	0.60	0.69	140
16 S rRNA	479	36.25	10.12	16.91	36.71	61	0.57	0.61	156
Combined Sequence	803	35.64	10.21	17.35	36.79	103	0.57	0.63	224

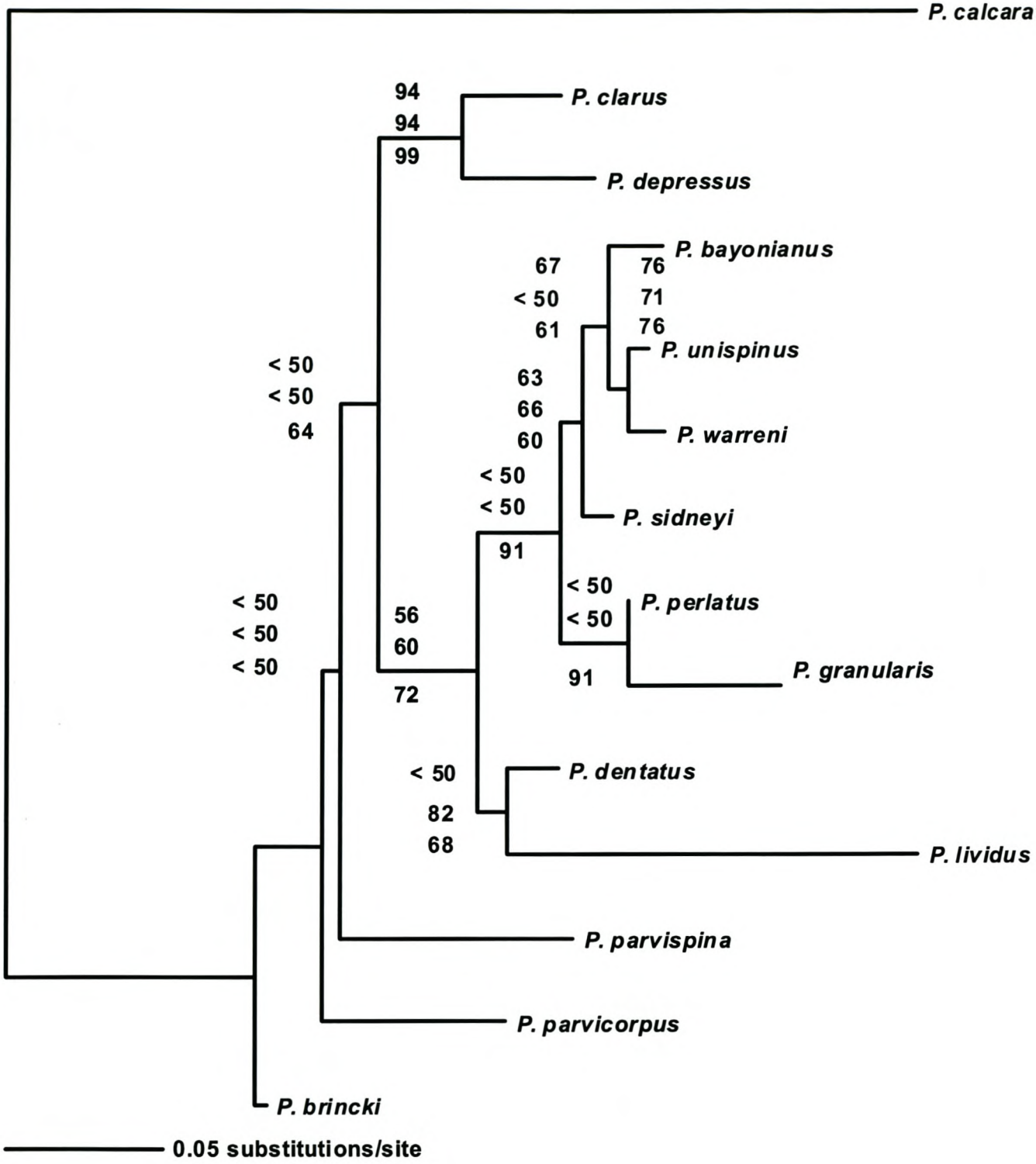


Figure 8. 3. The ML tree for the 16 S rRNA gene for the 14 southern African river crab species. Bootstrap value > 50% are shown on the branches. Values above the branch are for MP and NJ, while the value below is for ML. Branch lengths are shown proportional to the amount of change along the branch.

Combined sequence analysis

The GTR + Γ model was selected for ML. A different estimated rate was used for each of the six variables ($R_1 = R_3 = R_4 = R_6 = 1.00$; $R_2 = 7.03$; $R_5 = 4.04$). The estimated shape parameter for the gamma distribution of the data was $\alpha = 0.2946$. Incorporating this model of molecular evolution, a phylogenetic relationship was estimated using maximum likelihood (-ln L = 3090.5737, fig. 8. 4). The ML tree provided strong support for the monophyly of the large bodied riverine species with 80 % bootstrap support. The ML tree also corroborated the relationship between the three species that possesses carapace dentition (*P. warreni*, *P. unispinus* and *P. bayonianus*) with 92 % bootstrap support. The NJ search recovered a tree topology identical to the ML and MP trees, thus strongly supporting relationships derived from the combined sequence analysis. In addition, the sister taxon relationship between *P. warreni* and *P. unispinus*, *P. perlatus* and *P. granularis* and *P. clarus* and *P. depressus* were always recovered. The remainder of the nodes were poorly supported.

Results from the partition homogeneity test were not congruent ($P = 0.068$ for parsimony uninformative characters included, and $P = 0.439$ for parsimony uninformative characters excluded). The topology strongly supports the relationships between the large bodied riverine species (72 %). The sister taxon relationship between *P. perlatus* and *P. granularis* (99 %). The analysis also included *P. dentatus* and *P. lividus* as sister taxon to the large bodied river crabs. Again, as with the separate analysis of the two gene fragment, the combined analysis also recovered the sister taxon relationships between *P. depressus* and *P. clarus* and between *P. brincki* and *P. parvicorpus* with bootstrap support of 99 % and 86 % respectively.

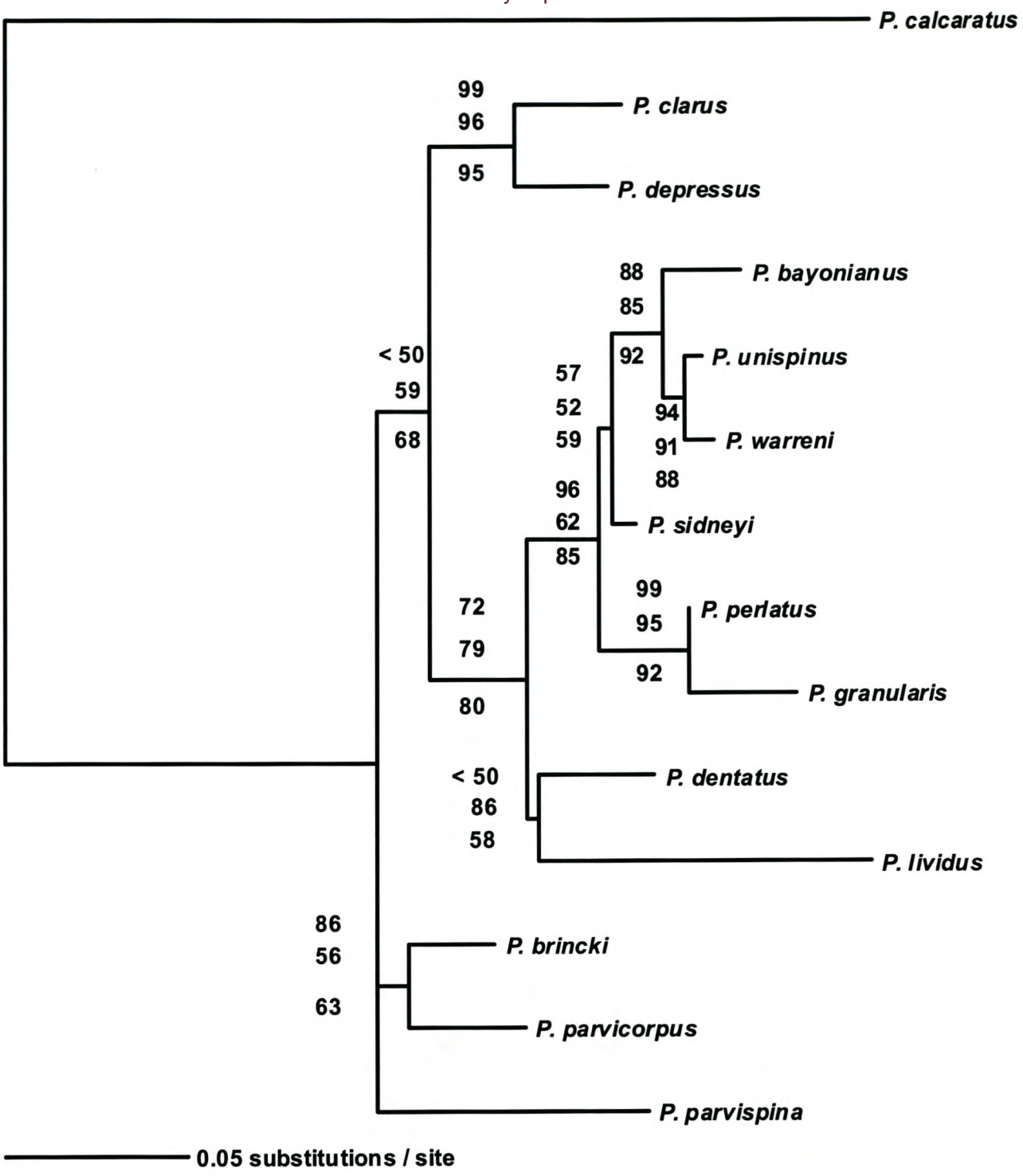


Figure 8. 4. The ML tree for the combined sequence (12 S rRNA and 16 S rRNA) data for the 14 southern African river crab species. Values above the branch are for MP and NJ, while the value below is for ML. Branch lengths are shown proportional to the amount of change along the branch.

The mountain stream species found in the Western Cape (*P. brincki, P. parvicorpus* and *P. parvispina*) are basal in this analysis and do not appear to be closely related to the large bodied riverine species.

Topological constraints

Bott's (1955) subgeneric divisions for the *Orthopotamonautes* (*P. depressus* and *P. sidneyi*) and the *Potamonautes* (*P. perlatus* and *P. warreni* and *P. bayonianus*) was tested, by constraining the monophyly of species belonging to the same subgenus. Similarly, the biogeographic relationships between mountain stream species and riverine species were examined by enforcing the monophyly of sympatric non hybridizing species. For this comparison, *P. perlatus* and *P. brincki* was constrained as a monophyletic entity and likewise *P. perlatus* and *P. parvicorpus*. The Shimodaira-Hasegawa (1999) test indicated no support for either the subgeneric divisions or the biogeographic relationship among sympatric mountains stream and riverine taxa (table 8. 6).

Sequence divergence

For the 12 S rRNA gene fragment, sequence divergence based on the ML model ranged from a minimum of 0.65 % between *P. perlatus* and *P. granularis*, to a maximum of 43.40 % between *P. calcaratus* and *P. brincki*. For the 16 S rRNA gene, sequence divergence values based on the ML model ranged from a minimum of 1.65 % between *P. warreni* and *P. unispinus*, to a maximum of 27.78 % between *P. lividus* and *P. parvispina*. A lower mean sequence divergence value was detected among hybridizing species (12 S rRNA, mean = 2.71 %; 16 S rRNA, mean = 4.35 %),

compared to non hybridizing sympatric species pairs (12 S rRNA, mean = 8.62 %; 16 S rRNA, mean = 12.21 %). A comparison of the average sequence divergence between sympatric hybridizing and sympatric non hybridizing species (table 8. 7) showed that on average, hybridizing species pairs had significantly lower sequence divergence values between species pairs (t = -3.44, df = 9, P = 0.007 for 12 S rRNA; t = -3.687, df = 9; P = 0.005 for 16 S rRNA).

Morphology

Maximum parsimony analysis of the morphological data yielded 21 trees with 44 steps (CI = 0.47, RI = 0.61). These trees were constructed with 17 (65 %) parsimony informative characters. A 50 % majority rule consensus tree (fig. 8. 5) recovered a group that comprised all the small bodied mountain stream species (*P. depressus*, *P. clarus*, *P. brincki* and *P. parvicorpus*) with the remaining small bodied freshwater species *P. parvispina* falling in a group with *P. dentatus* and *P. bayonianus*. In addition, the consensus tree also recovered a second group of large bodied species that contained *P. perlatus, P. sidneyi, P. granularis*, *P. warreni* and *P. unispinus*. A 1000 bootstrap replicates of the data recovered three weakly supported clades (fig. 8. 6). Clade one comprised the small bodied mountain stream species (except *P. parvispina*) however, bootstrap support for this clade was weak (50 %). In this clade *P. depressus* and *P. clarus* are sister taxa (55 %). The second clade comprised *P. granularis*, *P. warreni* and *P. unispinus* (66 %). The third clade recovered by the morphological data comprised *P. bayonianus* and *P. dentatus* (53 %).

Table 8. 6. Statistical support for the alternative hypothesis on *Potamonautes* phylogenetic relationships based on the Shimodaira-Hasegawa test (SH test). Bott's (1955) subgenera are marked with superscript, while biogeograhic constrained hypothesis are unmarked.

Tree	-ln L	Δ-ln L	SH test *P* - value
unconstrained tree	3090.5737		
P. sidneyi sister to *P. depressus*[1]	3163.360	71.76	0.000
P. perlatus sister to *P. warreni* and *P. bayonianus*[2]	3155.960	65.39	0.000
P. perlatus sister to *P. brinck i*	3203.671	113.09	0.000
P. perlatus sister to *P. parvispina*	3175.860	85.28	0.000

Table 8. 7. Sequence divergence (based on the respective correction method inferred from likelihood analysis) data for sympatric species pairs for the two gene sequences.

Sympatric species pairs	12 S rRNA	16 S rRNA
Hybridizing species		
P. perlatus and *P. sidneyi*	3.26 %	3.50 %
P. granularis and *P. perlatus*	0.65 %	4.55 %
P. sidneyi and *P. unispinus*	3.66 %	2.55 %
P. clarus and *P. depressus*	3.29 %	6.83 %
Non hybridizing species		
P. perlatus and *P. parvispina*	14.24 %	14.20 %
P. perlatus and *P. brincki*	11.75 %	8.92 %
P. perlatus and *P. parvicorpus*	7.62 %	14.12 %
P. lividus and *P. sidneyi*	5.52 %	17.99 %
P. clarus and *P. sidneyi*	8.13 %	11.07 %
P. depressus and *P. sidneyi*	6.03 %	13.28 %
P. dentatus and *P. sidneyi*	7.05 %	5.92 %

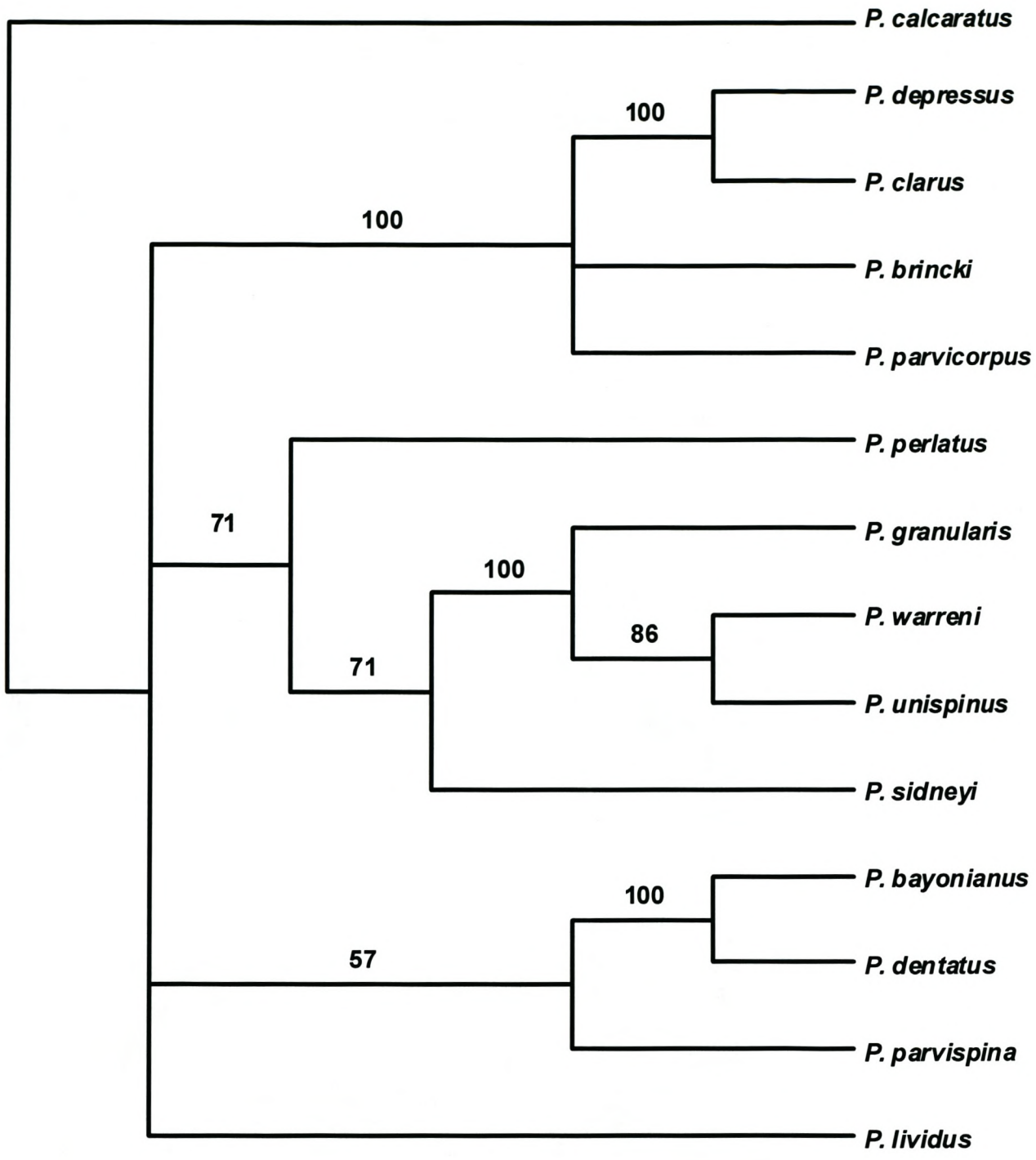


Figure 8. 5. A 50% majority rule consensus tree for the 21 trees derived from the morphological data.

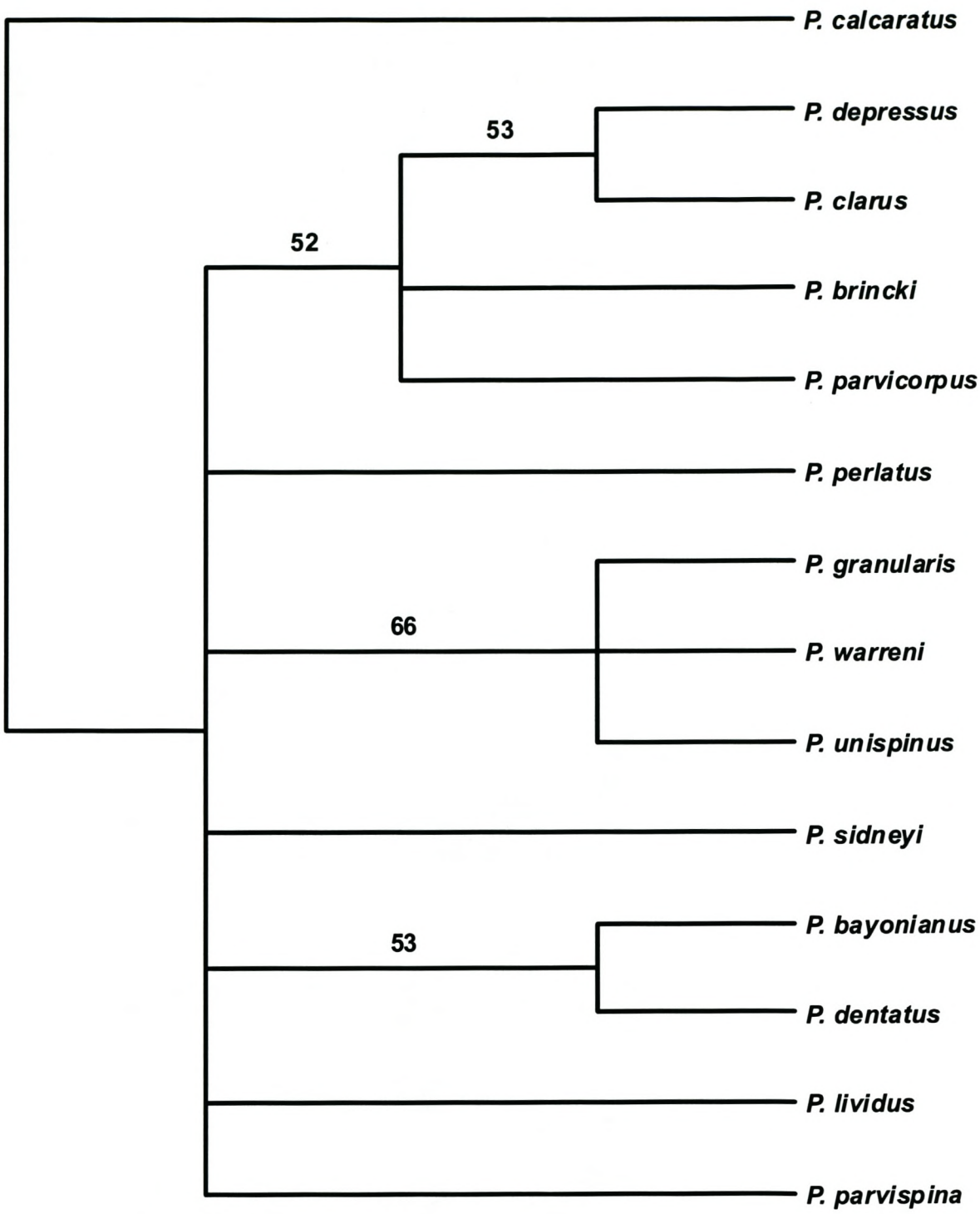


Figure 8. 6. The bootstrapped MP tree derived from the morphological data set. Bootstrap value > 50% are shown on the tree.

Successive weighting of the morphological data, produced 27 trees with a tree length of 119 steps (CI= 0.70; RI= 0.82). The bootstrapped tree however, revealed that the mountain stream species (*P. depressus, P. clarus, P. brincki* and *P. parvicorpus*) are closely related with 91 % bootstrap support, with the swamp forest species, *P. lividus* being their sister taxon (72 %).

Allozyme analysis

Allele frequencies are presented in Appendix 6. All the loci examined were polymorphic in at least one species and levels of polymorphism ranged from 0.00 to 28.6 %. The overall genetic variation as assessed using the observed heterozygosity, ranged from 0.005 to 0.046. This low variability is characteristic of crustaceans (Daniels *et al.*, 1998a, 1999a). All four distance methods used (Rogers, 1972; modified Rodgers (Wright, 1978), Prevosti (Wright, 1978) and unbiased Nei's distances (Nei, 1978) recovered the same topology, hence only a UPGMA tree based on Nei's distances is presented (fig. 8. 7).

This phenogram recovered the relationship between three mountain stream species in the Western Cape (*P. parvispina*, *P. parvicorpus* and *P. brincki*), the genetic identity value (*I*) that separates these three species was 0.77. The next cluster recovered by the data, placed *P. warreni* and *P. unispinus* as sister taxa ($I = 0.92$), in a cluster with *P. dentatus*. Interestingly, these are all large riverine species with carapace teeth on the anteriolateral margin.The following cluster placed *P. granularis* as the sister taxa to *P. perlatus* ($I = 0.94$), while the sister taxa of *P. sidneyi* is *P. lividus*. ($I = 0.84$). The sister taxon relationships between *P. clarus* and *P. depressus* is also recovered .

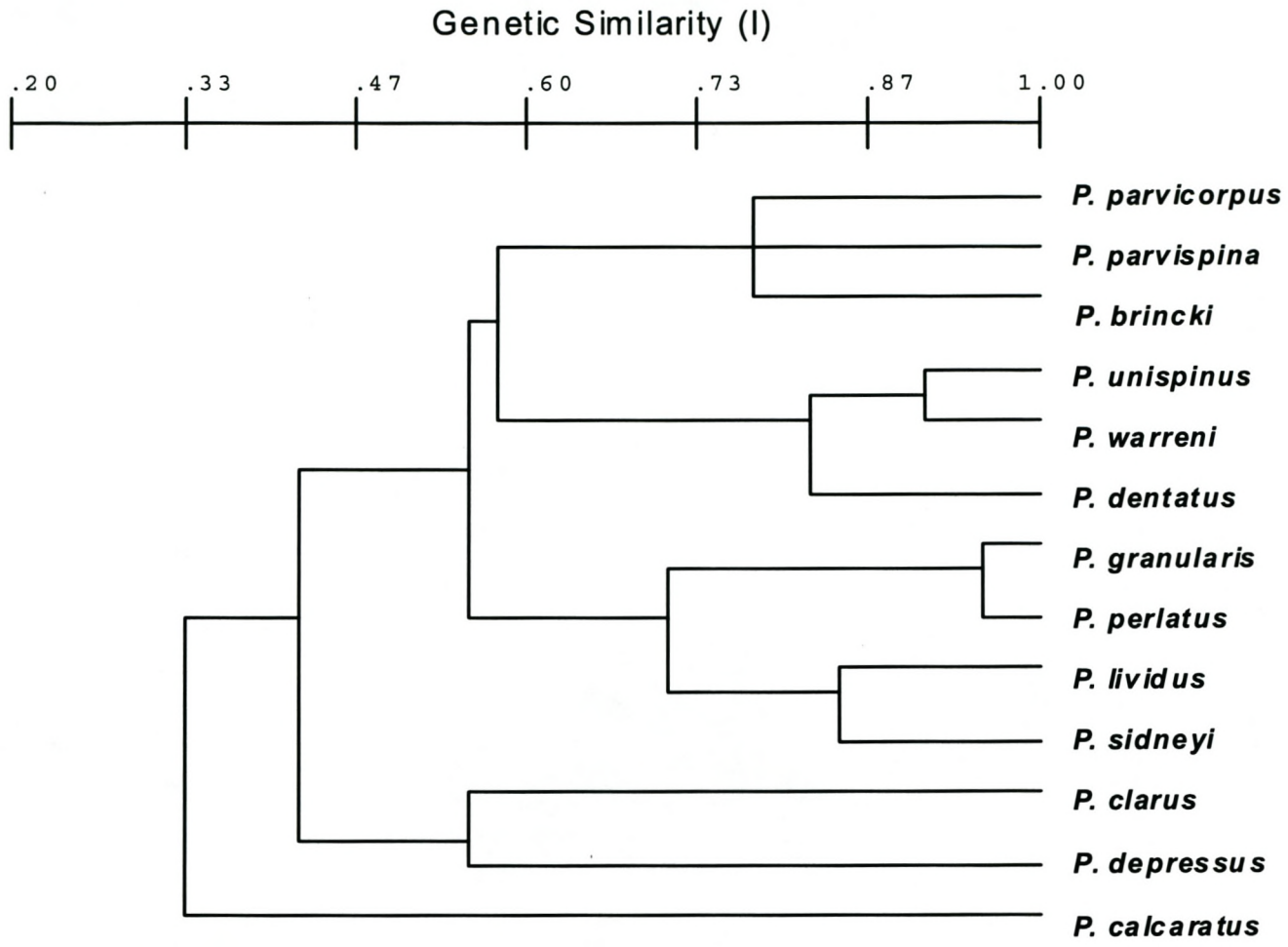


Figure 8. 7. Phenogram based on Nei's (1978) distance.

Potamonautes calcaratus appears to be the most divergent of all the species with a *I* of 0.35 separating it from the other clusters in the tree. This result corroborated the genetic distinctiveness of *P. calcaratus* thus further justifying its use as an outgroup for the *Potamonautes*.

Combinality test

Results of the partition homogeneity tests are presented in table 8. 8. Statistically there was no difference when uninformtaive characters were included or excluded for the IDL test (t = -1.015; df = 16; P = 0.324). These results are in contrast to Lee (2001) who argues for the exclusion of parsimony uninformative characters. Both tests either supported or rejected the combinality of data sets. Incongruence was detected between the two gene sequence data sets as well as the allozyme and morphology data sets ($P > 0.05$). All combinations of alternative data sets were congruent ($P < 0.01$). Data sets were combined into a single "total evidence" matrix.

Total evidence approach

Maximum parsimony analysis of the combined data sets (12 S rRNA, 16 S rRNA, morphology and allozymes) contained 108 parsimony informative characters and yielded two trees that were 279 steps long (CI = 0.53; RI =0.59). A bootstrapped MP tree (fig. 8. 8) supports the monophyly of the large bodied riverine freshwater species (72 %). The swamp forest species *P. lividus* is placed basal to the riverine species, *P. dentatus* (77 %). In addition, the phylogenetic relationship between the riverine species that are able to hybridize is well-supported (95 %). Furthermore the relationship between the three species with teeth on the anterolateral margins (*P.*

Table 8. 8. Results of the partition homogeneity tests for comparisons among all data partitions among all data partitioned. Tests with parsimony-uninformative characters 1 included and 2 excluded.

Comparison	*P* value[1]	*P* value[2]
12 S rRNA- allozymes	0.001	0.008
12 S rRNA- morphology	0.001	0.01
16 S rRNA- allozymes	0.001	0.001
16 S rRNA - morphology	0.001	0.002
12 S rRNA- 16 S rRNA	0.068	0.439
Combined DNA - allozymes	0.015	0.067
Combined DNA- morphology	0.001	0.002
Allozymes and morpology	0.133	0.156
All data sets	0.001	0.002

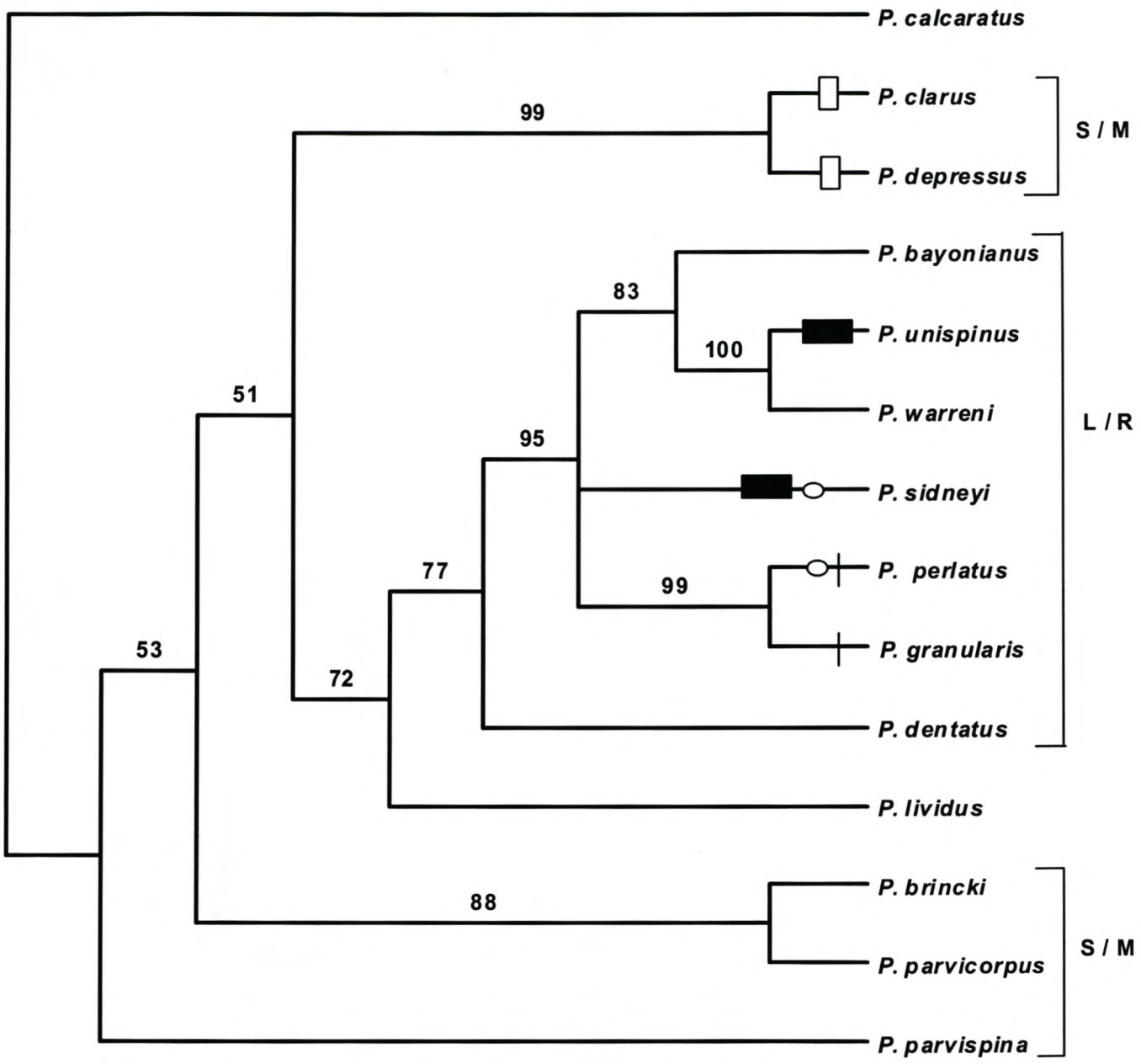


Figure 8. 8. The bootstrapped MP tree based on the "total evidence approach". Bootstrap value > 50% are shown on the tree. Taxa that are known to hybridise are marked with identical symbols. S/M are the small bodied mountain stream species, while L/R are the large bodied riverine species.

Rodriguez (1995b) hypothesis.The distribution of freshwater crabs on southern Africa has been influenced by factors such as geological and hydrographic changes. Interpretation of faunal distribution patterns in South Africa is complex, but is thought to be largely governed by climate, with the 16 ^{0}C isotherm dividing the country into two temperature regimes comprising a temperate and tropical faunal assemblage (Stuckenberg, 1962, 1969). These rough biogeographic boundaries seemed to have played a role in the evolutionary biology of freshwater crabs. The distribution of South African freshwater crab species bears testimony to the fact that temperature profiles govern distribution patterns of organisms. From our study it is clear that the tropical fauna comprised of toothed riverine species (*P. warreni*, *P. unispinus* and *P. bayonianus*) are confined to the area north of the Orange River system and include the Limpopo and Zambezi rivers and the Okavango delta (fig. 8. 1). These species appear to be phylogenetically closely related to other large bodied riverine species such as *P. sidneyi*, *P. perlatus* and *P. granularis*. Interestingly, the former two species have distribution patterns that commonly overlap with the tropical species. For example, both *P. perlatus* and *P. sidneyi* have been recorded in the Orange River system, and the large bodied freshwater crab species also included the subtropical KwaZulu-Natal endemics species, *P. dentatus* and *P. lividus*. It would be reasonable to hypothesize that these riverine species were derived from a widespread tropical riverine ancestral species. On the other hand, mountain stream species that occur in close geographic proximity are more closely related to each other (*P. brincki* and *P. parvicorpus*; *P. depressus* and *P. clarus*), however, on the whole, mountain stream taxa do not appear closely related and might have evolved at least twice. Once in mountains in the Western Cape and the secondly in the Drakensberg mountains. These temperate

mountains stream species are all endemic to South Africa.

In an attempt to explore possible vicariance events that could have contributed to the current distribution of the freshwater crab species a molecular clock is applied. However, the application of such clocks to our data remain tentative at best, considering the absence of fossil data that would allow for reasonably accurate dating of historic geological processes. Although a molecular clock for the 12S rRNA mt DNA gene fragment has been calibrated for arthropods (Brower, 1994), it appears to be quite variable, ranging from 2.3 % per MYR (million years) to 0.5 % per MYR (Lynch and Jarrell, 1993). However, for the 16S rRNA mt DNA gene the molecular clock appears remarkably stable, regardless of which decapod class is examined (Strumbauer *et al.,* 1996; Schubart *et al.*, 1998). A molecular clock of 0.90 to 0.88 % sequence divergence represents roughly 1% / MYR BP. Extrapolating the clock to our 16 S rRNA sequence divergence data, separation of the tropical species clade occurred 3 MYR, while the separation between the temperate species (Cape Fold and Drakenberg Mountain species) is placed at 12 MYR BP (+ - 2 MYR). Thus the speciation between the tropical species can be dated to the early Pliocene while the speciation between the temperate mountain stream faunal groups on the Cape Fold and Drakensberg Mountains most likely took place during the middle Miocene. The data from the present study are supported by hydrographic and geological evidence indicating that as recently as the Pleistocene there was a link between the Okavango delta and the upper Zambezi tributaries and these were connected to the Limpopo river. In turn, tributaries of the latter river system were connected to the upper Orange river (Moor, 1988). These river systems have changed their courses extensively in the

last million years, thus aiding the distribution and isolation of taxa. The Orange River was also connected to the Olifants River during the Miocene, and this would have facilitated the dispersal of the ancestral species (Partridge and Maud, 1987). In addition to these biogeographic events, climatic change probably also played an important role in the evolution of southern African river crab species. It is well documented that during the late Miocene the subcontinent underwent dramatic climatic changes that occilated between mesic and xeric cycles, largely due to glaciation cycles (Tyson, 1986; Coetzee, 1993). This scenario of drainage evolution is invoked to explain the distribution of the large bodied river crab species based on the degree of interconnection between river systems. This pattern of drainage evolution explains the phylogenetic relationship between *P. warreni*, *P. unispinus* and *P. bayonianus*. Interestingly, the distribution pattern found amongst the large bodied freshwater crabs is also mirrored amongst freshwater fishes (Skelton, 1994).

It has long been suspected that the freshwater crab fauna, as well as the freshwater fish fauna, is the result of invasions from northern African regions (Bott, 1955; Jubb and Farquharson, 1965; Kensley, 1981). These invasions were thought to have been facilitated by drainage connections. Phylogenetic results from the present study lend marginal support to this hypothesis, considering that the centre of origin (the area with greatest diversity where relict taxa exist) for *Potamonautes* is thought to have been the Congo basin (Cumberlidge, 1999a). However, considering that no species from the Congo basin were included in this study, the "north-south invasion hypothesis" cannot be rejected or confirmed with absolute confidence and requires further investigation. However, *P. calcaratus* is a species that occurs along the eastern escarpment from

Ethiopia into the Kruger Park in South Africa. The fact that this species was consistently placed basal may point to its antiquity. Phylogenetic analysis of the remaining southern African inland decapod fauna, such as the prawn genera *Caridina* and *Macrobrachium* should provide additional insight into the biogeographic distribution of freshwater organisms on the subcontinent and elucidate possible relationships with species further north.

In the present study, hybrid species pairs are indeed phylogenetically closely related based on the sequence divergence values and on their position in the phylogeny. This becomes apparent when relationships between *P. perlatus* and *P. granularis*; *P. perlatus* and *P. sidneyi* and *P. sidneyi* and *P. unispinus* are examined. These species are known to hybridize when occurring sympatrically and fall into the same clade, which is also supported by high bootstrap values. In addition, most species pairs that are capable of hybridization have low sequence divergence values compared to sympatric non-hybridizing species pairs. These results are suggestive of poorly developed pre / or postzygotic isolation mechanisms among hybridizing species. The prevalence of interspecific freshwater crab hybrids could be interpreted as indicative of a relatively young age for the fauna. It would also appear that among freshwater crab species, sequence divergence values are proportional to the ability to hybridize, hence species pairs characterized by low sequence divergence values are more likely to hybridize. Such results corroborate Smith's (1992) hypothesis that reproductive compatibility between species may not be blocked by subsequent evolution. These results imply that given the opportunity, freshwater crab species with low sequence divergence may hybridize. Freshwater taxa appears to be specifically prone to

hybridization, this could probably be attributed to the fact that conspecific allopatric freshwater taxa are less subject to selective divergence.

In the present study, the morphological data set provided limited resolving power in determining species relationships among the freshwater crabs primarily because of the high degree of convergence in adult morphology (Barnard, 1935, 1950). Limited phylogenetic inferences can thus be drawn from the morphological analysis, however the progression in body size is thought to have coincided with the transition from a large bodied riverine freshwater species to a species adapted for semi-terrestrial life. In the *Plathythelphusa,* Von Sternburg and Cumberlidge (1999b) recorded an inverse pattern suggesting that when discerning the progression of morphological characters for phylogenetic studies, characters should be evaluated individually to merit their use. In addition, such studies should incorporate more of the described *Potamonautes* species in an attempt to accurately assess the morphological diversity and evolution of characters. The sequence data and the combined data analyses presented here confirm that the southern African potamonautid crabs do not conform to Bott's (1955) subgeneric designations, based on morphology. For example, the species *P. depressus* and *P. sidneyi* that were placed in the subgenus *Orthopotamonautes* by Bott (1955) are found not to be sister taxa. Moreover, the results suggest that none of the remaining subgeneric divisions may be valid taxonomic entities. Noticeably, Bott's subgeneric divisions were largely fuelled by earlier work undertakings by Barnard (1935; 1950) who consistently reported the presence of transitional forms between species. For example, Barnard noted that the variation in carapace dentition pattern between *P. warreni* populations varied from a typical form with 5 to 10 well defined

teeth to a form that resembles *P. perlatus* which is characterized by a smooth to granulated anteriolateral margin. Based on these studies Bott (1955) assigned these two species together with *P. dubius* and *P. bayonianus* to the subgenus *Potamonautes*. Both Bott (1955) and Cumberlidge (1999a) perceived a primitive body plan for the freshwater crabs that included for example, large teeth on the anteriolateral carapace margins, an incomplete postfrontal crest and a flat carapace. It would however appear that many of these plesiomorphic characters may in fact be apomorphic characters indicating that character status needs radical reassessment. A similar sentiment was recently expressed by Von Sternburg and Cumberlidge (1999b) while working on the *Plathythelphusa*. Based on the placement of potamonautid species in the total evidence data set, it appears that the small mountain stream fauna represents the plesiomorphic body plan, while the large riverine species represents the apomorphic state. Results from this study clearly challenge Cumberlidges (1999a) hypothesis that the large bodied species represents the plesiomorphic condition. Fossil evidence may provide invaluable data to track the change in character states within *Potamonautes*. However, the fossil record for potamonautid crabs is sparse (Carriol and Secretan, 1992), hence the ontogeny of morphological characters in this group remains largely obscure. Cumberlidge (1999a) is also of the opinion that none of the smaller bodied riverine and semi - terrestrial species ever possesses teeth on the anteriolateral margin of the carapace. This statement does not hold true for the southern African freshwater crabs. For example, a small but distinct epibranchial tooth is evident in *P. parvispina* and *P. calcaratus*.

Homologous structures need to be defined for phylogenetic analysis, as homology is

crucial in cladistics (Bellwood, 1996, 1998). However, homoplasy of characters resulting from convergence and parallelism often obscures phylogenetic signal and compromises phylogenetic inferences. Systematic studies on brachyuran crabs have encountered evidence of substantial homoplasy in external adult morphology, such as carapace ornamentation and zoel morphology (e.g. Guinot, 1977; Rice, 1980). A similar sentiment can be expressed for the carapace variables used in the same morphological study. Harrison and Crespi (1999) concluded that for *Cancer* crab species, selective habitat pressure results in convergence in the overall size and shape of external morphological traits. Schubart *et al.,* (2000) recently suggested that convergence might be responsible for the phenotypic similarities between divergent mud crab lineages. Similar results are evident among the small-bodied mountain stream species, where species are generally characterized by smooth flat carapaces, a direct adaptation to stream life with the same ecological factors operating in these habitats. Numerous systematists have concluded that the accuracy of morphological trees can be significantly improved by including characters that are less subject to selective pressures, such as setae number, antennae, gonopod structures and soft anatomical structures (Jamieson, 1990; Abele, 1991; Harrison and Crespi, 1999). Future morphological studies should aim to include internal characters derived, for example, from sperm morphology and detailed histological studies of gonopod one. In light of such uncertainty, morphological characters should be sieved to determine their utility in phylogenetic analysis.

Overall, morphological diversity in the Southern African group seems to be constrained, and character evolution has rarely involved the emergence of dramatic

apomorphies. This problem is confounded by cryptic speciation in the *Potamonautes* (Daniels *et al.,* 1998a; Gouws *et al.,* 2000) thus further limiting the application of morphological characters. Stasis in morphological evolution is likely to be attributed to the success of the body architecture in decapod crabs and possibly reflects on the antiquity of crabs. A similar finding has also been documented among *Daphnia* species where Taylor *et al.*, (1998) reported that, for this group of freshwater crustaceans, morphological evolution was negligible, and conclude that morphological stasis is the "Gordonian knot of evolutionary biology".

Allozyme data has been extensively used in phylogenetic studies in a wide range of organisms (for a review see Thorpe and Sole-Cava, 1994). However, Fetzner (1996) concluded that for freshwater crayfish (*Orconectes*) and possibly other decapod Crustacea, the technique might have limited application at subgeneric levels due to the limited level of polymorphism and low heterozygosity. Fetzner (1996) further remarked that these factors resulted in poorly resolved phylogenies that restricted phylogenetic inferences. Studies conducted to date suggest that levels of polymorphism and heterozygosity within conspecific freshwater crab populations are generally low and the resolution of relationships would thus not benefit from including more populations or larger samples (Daniels *et al.,* 1999). Interspecific genetic identity values for most species pairs compare favourably with those reported in previous studies (e.g. Daniels *et al.,* 1999; 2001), with most species having *I* values that range from 0.35 to 0.85, and only two species pairs having *I* values > 0.85. In the present instance, the allozyme data generally performed poorly, largely due to the limited number of loci examined.

Avise (1994) concludes that where congruent phylogenetic patterns are derived from independent sources of data (mtDNA gene, nuclear trees and morphology) the species tree would be more accurately reflected. In the present study some congruence was observed between the different data sets examined, with the combined sequence data recovering a tree topology virtually identical to that of the "total evidence" topology. Although the use of multiple gene genealogies, such as in this study, generally yield more accurate phylogenies relative to the use of a single gene, the use of two mt rRNA genes should be considered a starting point in determining species relationships.

Analysis of the sequence data sets failed to resolve several of the internal nodes in the tree possibly because of the rapid radiation as evident from the short branches in the tree. This study could benefit from using additional data (possibly nuclear gene) as it appears that speciation events occurred relatively rapidly hence the conserved rRNA genes may not be the most suitable genes to investigate this phenonomen. Matthee *et al.,* (2001) recently demonstrated that the use of nuclear sequence data is proving to be generally more informative at recovering phylogenetic relationships where mtDNA sequence has had limited resolving power. While the objectives of the present study were met using the limited taxonomic sampling, future studies that are to construct a phylogeny for *Potamonautes* should aim to incorporate better taxonomic sampling (Bremer *et al.*, 1999; Barraclough and Nee, 2001). In addition such studies should aim to include morphological characters derived from soft anatomical features that are supposedly less subject to convergence and include sequence data from additional molecular markers.

Chapter 9

Summary

The present study highlights the fact that the freshwater crab fauna of southern Africa is considerably more species rich than previously thought. Since the inception of the study on freshwater crab systematics nearly ten years ago, at least six new species have been described, and a new species, *Potamonautes parvicorpus*, from mountain streams on the Cape Peninsula is described in the present study (chapter 3). These systematic results demonstrate that while most species were thought to have large distribution ranges, a large number of species appear endemic to South Africa. There has undoubtedly been a trend from widely distributed species to species with narrow distributions. The results contradict previously published reports that certain species such as *P. perlatus* and *P. sidneyi* have broad geographic distribution ranges. The discovery of cryptic species among these widespread taxa further points to the fact that genetic studies are needed to assess species boundaries among freshwater crabs. Detailed genetic analysis of areas further afield will undoubtedly continue to demonstrate the presence of new undescribed taxa.

Where systematic affinities among taxa are unclear due to varying levels of phenotypic plasticity in diagnostic characters, genetic data, particularly allozymes, proves useful to discern species boundaries. For example, an investigation into the systematic status of two taxa with variable carapace dentition, *P. warreni* and *P. unispinus* using genetic data demonstrated that the two taxa are indeed distinct species (chapter 4). The systematic value of dentition patterns is explored. Systematically, carapace dentition patterns appear to be valuable in species diagnosis in freshwater

crabs. Caution should however be exercised where carapace dentition patterns are highly variable. In such instances, features of pleopod 1 should be examined in concert with the mandibular palp to determine species status. Evident from the chapter four was that the original decription of *P. warreni* is poor. Hence, the holotype is re-described, the diagnostic features of the species is examined and compared to other southern African freshwater crabs in *Potamonautes* (chapter 5) while the degree of morphological variation is quantified.

Freshwater crabs were thought to hybridize extensively based on the high degree of morphological variation between species. Genetic research on species has demonstrated that hybridization in infrequent and restricted to four species pairs (*P. perlatus* and *P. granularis*; *P. perlatus* and *P. sidneyi*; *P. sidneyi* and *P. unispinus* and *P. depressus* and *P. clarus*). Most hybrid zones are narrow. In the present study, hybridization, in two freshwater crab species, *P. depressus* and *P. clarus* is examined (chapter 6). Results from this study indicate that at least five evolutionary significant units could be identified using genetic markers. Furthermore, these results demonstrate that taxa can arise through hybridization and that these taxa deserve equal conservation effort. Arguments for the conservation of hybrid taxa are made.

The population genetic structure of numerous freshwater crab species has been examined to date. Conspecific populations are generally genetically invariant, and the genetic differentiations between populations that occur in close proximity are negligible. In the present study (chapter 7) the population genetic structure of the burrowing freshwater crab, *P. calcaratus* is examined using sequence and allozyme

data. Results revealed that populations were genetically structured and that moderate to limited gene flow is occurring and that most populations are the result of recent separation. These results are compared with data from other freshwater crabs species.

With the determination of species boundaries of *Potamonautes* in southern Africa nearing completion, the phylognetic relationships among taxa were examined. Phylogenetic relationships among the southern African freshwater crab fauna have not been previously investigated. In the present study, multiple data sets (allozymes, sequencing and morphology) are used to firstly, examine the use of freshwater crabs as indicators of biogeography, to secondly, examine the relationship between hybridizing species pairs and to thirdly examine the subgeneric divisions proposed by Bott (1955). The results show that freshwater crabs are indeed suitable zoobiogeographic indicators and placed all the large bodied species into a distinct clade, while mountain stream species was more closely related to sister taxa that occurred in the same geographic region. Hybrid taxa are indeed phylogenetically closely related and Bott's subgenera have no systematic value. The genetic data sets performed well, while the morphological data poorly resolved relationships probably due to convergence and selection. Future studies should aim to incorporate additional sequence data in an attempt to resolve the deeper nodes of the tree topology.

References

Abele, L. G. (1991). Comparison of morphological and molecular phylogeny of the Decapods. *Memoirs of the Queensland Museum,* **31:** 101-108.

Abell'o, P., Pertierra, J. A. and Reid, D. G. (1990). Sexual size dimorphism, relative growth and handedness in *Liocarcinus depurator* and *Macropipus tuberculatus* (Brachyura: Portunidae). *Scientia Marina*, **54:** 195-202.

Abby- Kalio, N. J. and Warner, G.F. (1989). Heterochely and handedness in the shore crab *Carcinus maena* (L) (Crustacea: Brachyura). *Zoological Journal of the Linnean Society,* **96:** 19-26.

Alcock, A. (1909). Diagnoses of species and varieties of freshwater crabs. Nos. 1-4. *Records of the Indian Museum,* **3:** 243-252.

Alcock, A. (1910a). Brachyura I. Fasc. II The Indian Freshwater Crabs – Potamonidae. Catalogue of the Indian Decapod Crustacea in the collection of the Indian Museum. Calcutta, pp. 1-135.

Alcock, A. (1910b). On the classification of Potamonidae (Telphusidae). *Records of the Indian Museum,* **5:** 253-261.

Allegrucci, G., Caccone, A., Cataudella, S., Powell, J. R. and Sbordoni, V. (1995). Acclimation of the European sea bass to freshwater: Monitoring genetic changes by

RAPD polymerase chain reaction to detect DNA polymorphisms. *Marine Biology,* **121:** 591-599.

Allanson, B. R., Hart, R. C., O'Keeffe, J. H. and Robarts, R. D. (1990). Inland Waters of Southern Africa: An Ecological Perspective. *Monographiae Biologicae*, **64:** 1-458.

Aotsuka, T., Susuki. T., Moriya. T. and Akemi, I. (1995). Genetic differentiation in Japanese freshwater crab, G*eothelphusa dehaani* (white): Isozyme variation among natural populations in Kanagawa Prefecture and Tokyo. *Zoological Science,* **12:** 427-434.

Armbruster, G. (1997). Evaluations of RAPD markers and allozymes patterns: evidence for morphological convergence in the morphotypes of *Cochlicopa lubricella* (Gastropoda: Pulmonata: Cochlicopidae). *Journal of Molluscan Studies,* **63:** 379-388.

Avise, J. C. (1974). Systematic value of electrophoretic data. *Systematic Zoology,* **23:** 465-481.

Avise, J. C. (1976). Genetic differentiation during speciation. In: Ayala, F. J. (ed.). *Molecular Evolution.* Sinauer Associates, Inc., Sunderland, Massachusetts. Pp. 106-122.

Avise, J. C. (1989). A role for molecular genetics in the recognition and conservation of endangered species. *Trends in Ecology and Evolution*, **4:** 279-281.

Avise, J. C. (1994). Molecular Markers, Natural History and Evolution. Chapman and Hall, New York, U.S.A.

Avise, J. C. (1995). Mitochondrial DNA polymorphism and a connection between genetics and demography of relevance to conservation. *Conservation Biology*, **9:** 686-690.

Balss, H. (1957). Decapoda, VIII: Systematik. In. H G. Bronn, Klassen und Ordnungen des Tierreichs, Bd 5, Abteilung 1, **7:** 1505-1672.

Banarescu, P. (1990). Zoogeography of fresh waters, 1. General distribution and dispersal of freshwater animals, 1- 511. (AULA- Verlag Wiesbaden)

Barbaresi, S., Gherardi, F. and Vinnini, M. (1997). Movement patterns of river crabs (Decapoda, Potamoidea) in the field: predictable and unpredictable components. *Journal of Zoology London,* **242:** 247-259.

Barber, P. H. and Erdmann, M. V. (2000). Molecular systematics of the Gonodactylidae (Stomatopoda) using mitochondrial cytochrome oxidase C (Subunit 1) DNA sequence data. *Journal of Crustacean Biology*, **20:** 20-36.

Bardakci, F. and Skibinski, D. O. F. (1994). Application of the RAPD technique in tilapia fish: species and subspecies identification. *Heredity,* **73:** 117-123.

Barnard, K. H. (1935). Scientific results of the Vernay-Lang Kalahari expedition, March to September, 1930. *Annals of the Transvaal Museum,* **16:** 481-492.

Barnard, K. H. (1950). Descriptive catalogue of South African decapod Crustacea (crabs and shrimps). *Annals of the South African Museum,* **38:** 1-837.

Barraclough, T. G and Nee, S. (2001). Phylogenetic speciation. *Trends in Ecology and Evolution,* **16:** 391-399.

Barton, N. H. and Hewitt, G. M. (1985). Analysis of hybrid zones. *Annual Review of Ecology and Systematics,* **16:** 113-148.

Barton, N. H and Hewitt, G. M. (1989). Adaptation, speciation and hybrid zones. *Nature,* **341:** 497-503.

Bas, C. and Sarda, F. (1998). Long term morphometric variation in a population of the deep-sea shrimp, A*risteus antennatus* (Risso, 1816) (Decapoda; Aristeidae). *Crustaceana,* **71:** 369-377.

Bellwood, O. (1996). A phylogenetic study of the Calappidae (Crustacea: Brachyura) with a re-appraisal of the status of the family. *Zoological Journal of the Linnaen Society*, **18:** 165-193.

Bellwood, O. (1998). The phylogeny of the box crab genera (Crustacea: Brachyura:

Calappidae) with notes on their fossil record, biogeography and depth distribution. *Journal of Zoology London*, **244:** 459-471.

Benzie, J. A. H., Frusher, S. and Ballment, E. (1992). Geographic variation in allozyme frequencies of *Penaeus monodon* (Crustacea: Decapoda) populations in Australia. *Australian Journal of Marine and Freshwater Research*, **43:** 715-725.

Benzie, J. A. H. and Stoddart, J. A. (1992). Genetic structure of crown of thorns starfish (*Acanthaster placi*) in Australia. *Marine Biology,* **112:** 631-639.

Bert, T. M. (1986). Speciation in western Atlantic stone crabs (genus: *Menippe*); the role of geological processes and climatic events in the formation and distribution of species. *Marine Biology,* **93:** 157-170.

Bert, T.M., McCarthy K. J, Cruz-Lopez, H. and Bogdanowicz, S. (1996). Character discrimination power, character-set congruence and the classification of individuals from hybrid zones: An example using stone crabs (*Menippe*). *Evolution*, **50:** 655-671.

Bigelow, R. S. (1965). Hybrid zones and reproductive isolation. *Evolution,* **19:** 449-458.

Bilton, D. J, Goode, D. and Mallet, J. (1999). Genetic differentiation and natural hybridization between two morphological forms of the common woodlouse, *Oniscus asellus* Linnaeus 1758. *Heredity*, **82:** 462-469.

Birky, C W., Fuerst, P. and Maruyama, T. (1989). Organelle genetic diversity under migration, mutation, and drift: equilibrium expectations, approach to equilibrium, effects of heretoplasmic cells, and comparison to nuclear genes. *Genetics,* **21:** 613-627.

Black-Sameulsson, S., Ericksson. G., Gustafsson, L. and Gustafsson, P. (1997). RAPD and morphological analyses of the rare plant species *Vicia pisiformes* (Fabacea). *Biological Journal of the Linnean Society,* **61:** 325-343.

Bock, W. J. (1992). The species concept in theory and practice. *Zoological Science,* **9:** 697-712.

Bott, R. (1955). Die Susswasserkrabben von Africa (Crustacea: Decapoda) und ihre Stammesgeschichte. *Anales du Musee Royal du Congo Belge, Tervuren, Zoologie,***1:** 209-352.

Bott, R. (1960). Crustacea (Decapoda): Potamonidae.-In: B.Hanstrom, P.brinck, and G.Rudebeck, (eds). South African Animal life. *Results of the Lund University Expedition* in 1950-1951,**7:**13-18.

Bott, R. (1965). Die susswasserkrabben von Madagaskar (Crustacea, Decapoda). *Bulletin du Museum National d'Histoire Naturelle,* **2:** 335-350.

Bott, R. (1969). Subwasserkraben von Sud-Amerikas, und ihre Stammesgeschichte. *Abhandlungen der senckengergischen naturforschenden Gesellschaft,* **518:** 1-94

Bott, R. (1970a). Die Subwasserkrabben von Europa, Asien, Australien und ihre Stammesgeschichte. Eine revision der Potamoidea under der Parathelpusoidea (Crustacea: Decapoda), *Abhandlungen der senckenbergischen natureforschendedn Gesellschaft*, **526:** 1-338.

Bott, R. (1970b). Betrachtungen uber die Entwicklungsgeschichte und Verbreitung der Susswasser-Krabben nach der Sammlung des Naturhistorrischen Museum in Genf/Schweiz. *Reveu Suisse de Zoologie,* **77:** 327-344.

Bouvier, E. L. (1917a). Sur la classification des Eupotamonae, crabes d'eau douce de la famille des Potamonides. *Comptes-rendus de l'Academie des Sciences, Paris,* **165:** 613-621.

Bouvier, E. L. (1917b). Sur la classification des Parapotamonae, crabes d'eau douce de la famille des Potamonides. *Comptes-rendus de l'Academie des Sciences, Paris,* **165:** 657 – 659.

Bouvier, E. L. (1921). Decapoda. In: Voyage de Ch. Alluaud et R. Jeannel en Afrique oriental (1911-1912). *Resultats scientifiques. Crustaces, III Paris*: 23-62.

Brasher, D. J., Ovenden, J. R. and White, R., W. G. (1992). Mitochondrial DNA variation and phylogenetics relationships of *Jasus* spp. *Journal of Zoology London*, **227:** 1-16.

Bremer, B. R. K., Jansen, B., Oxelman, M., Backlund, H., Lantz, P. and Kim, K. (1999). More characters or more taxa for a robust phylogeny – case study from the coffee family (Rubiaceae). *Systematic Biology,* **48:** 413-435.

Brito-Capella, F. de. (1864). Descricao de tres especies novas de crustaceos de Africa occidentale observacoes a cerca do *Penaeus bacagei* Johnson, especie nova dosmares de Portugal. *Memorias de Academia Real dos Sciencias de Lisboa,* **3:** 1- 11.

Brito-Capella, F. de. (1871). Alguman especies novas on pouco conheudas de crustaceos pertencentes aos generos Calappae Telphusa. *Jornal de Sciencias Mathematicas, Physicas e Naturaes*, **3:** 128-134.

Brito-Capella, F. de. (1873). Descricao d'uma nova specie de Thelphusa d'Africa occidental. *Jornal de Sciencias Mathemaicas, Physicas e Naturaes*, **4:** 254-257.

Brower, A. V. Z. (1994). Rapid morphological radiation and convergence among races of butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proceeding of the National Academy of Science, USA.* **91:** 6491-6495.

Brown, W. M. (1985). The mitochondrial genome of animals. In: R.J. MacIntyre (ed). Molecular evolutionary genetics, Plenum, New York. pp.95-130.

Bucklin, A., Frost, B.W. and Kocher, T. D. (1995). Molecular systematics of three species of *Calanus* and three species of *Metridia* (Copepoda: Calanoida). *Marine*

Biology, **121:** 655-664.

Bucklin, A. and Kocher, T. D. (1996a). Source regions for recruitment of *Calanus finmarchicus* to Georges Bank; Evidence from molecular population genetic analysis of mt DNA. *Deep-Sea Research* II, **43:** 1665-1681.

Bucklin, A., Sundt, R. and Dahle, G. (1996b). The population genetics of *Calanus finmarchicus* in the North Atlantic. Proceedings of the ICES Workshop for a Trans Atlantic study of *Calanus finmarchicus. Ophelia*, **44:** 29-45.

Bucklin, A., Smolenack, S. D., Bentley, S. and Wiebe, P. (1997). Gene flow patterns of the euphausid, *Meganyctiphanes norvegica*, in the NW Atlantic based on mt DNA sequences for cytochrome b and cytochrome oxidase 1. *Journal of Plankton Research*, **19:** 1763-1781.

Burgener, M., Snyman, N. and Hauck, M. (2001). Towards a Sustainable Wildlife trade: an analysis of nature conservation legislation in South Africa with particular reference to the wildlife trade. Institution of Criminology, University of Cape Town.

Busack, C. A. (1988). Electrophoretic variation in red swamp (*Procambarus clarkii*) and White River crayfish (*P. acutus*) (Decapoda: Cambaridea). *Aquaculture,* **69:** 211-226.

Calman, W. J. (1918). A new river crab from the Transvaal. *Annual Magazine of*

Natural History, **9:** 234-236.

Campton, D. E., Berg, C. J., Robinson, L. M. and Glazer, R. T. (1992). Genetic patchiness among populations of queen conch (*Strombus gigas*) in the Florida Keys and Bimim. *Fisheries Bulletin, U.S.A,* **90:** 250-259.

Cornew, S. (1990). Aspects of the biology of the Cape River crab *Potamonautes perlatus* (M. Edw) with particular reference to feeding biology. B. Sc (Hons) thesis. University of Cape Town.

Carriol, R and Secretan, S. (1992). Presence dans le Bassin du Lac Albert (Ouganda) des le Miocene superieur, de *Potamonautes* (*Acanthothelphusa*) *niloticus* (Crustacea, Brachyura). *C. R. Acad. Sci. Paris, t.* 314, *Paleontology, Series,* **2:** 411-417.

Chace, F. A. (1942). Scientific results of a fourth expedition to forested areas in eastern Africa. III Decapoda: Crustacea. *Bulletin of the Museum of Comparative Zoology*, **91:** 185-233.

Chiba, S. (1997). Novel color polymorphisms in a hybrid zone of *Mandarina* (Gastropoda: Pulmonata). *Biological Journal of the Linnean Society,* **61:** 369-384.

Chu, K. H, Lee, S. C. and Tsoi, S. C. M. (1990). Enzyme polymorphism in three species of *Metanephrops* (Decapoda; Nephropidae) from Taiwan. *Biochemical Systematics and Ecology*. **18:** 555-558.

Chu, K. H. (1999). Morphometric analyses and reproductive biology of the crab *Charybdis affinis* (Decapoda, Brachyura, Portunidae) from the Zhujiang estuary, China. *Crustaceana*, **72:** 647-658.

Cheung, T. S. (1976). A biostatistical study of the functional consistency in the reversed claws of the adult male stone crabs, *Menippe mercenaria* (Say) *Crustaceana*, **31:** 137-144.

Christiansen, B., Pfannkuche, O. and Thiel, H. (1990). Vertical distribution and population densities of the necrophagous amphipod, *Eurythenes gryllus*, in the West European basin. *Marine Ecology Progressive Series*, **66:** 35-45.

Clayton, D. A. (1990). Crustacean allometric growth: A case for Caution. *Crustaceana*, **58:** 270-290.

Coetzee, J. A. (1993). African flora since the Jurassic. Pp. 37-61, in Goldblatt, P (ed). Biological Relationships between Africa and South America. Yale Univ. Press, New Haven, USA.

Colbourne, J. K and Hebert, P. D. N. (1996). The systematics of North American *Daphnia* (Crustacea: Anomopoda): A molecular phylogenetic approach. *Philosophical Transactions of the Royal Society of London, series B,* **351:** 349-360.

Colosi, G. (1920). I Potamonidi onservati del R. Museo Zoologico di Torino.

Bolletino dei Musei di Zoologia ed Anatomica comparata della R. Universita di Torino, **35:**1-39

Cox, A. J. and Hebert, P. N. (2001). Colonization, extinction, and phylogeographic patterning in a freshwater crustacean. *Molecular Ecology,* **10:** 371-386.

Cracraft, J. (1989). Speciation and its ontology. The empirical consequences of alternative species concepts for understanding patterns and process of differentiation. In: Otte, D, Endler, J.A. eds. *Speciation and its consequences*. Sunderland, M. A: Sinauer Associates, Inc.

Cracraft, J. (1997). Species concepts in systematics and conservation biology- an ornithological viewpoint. In Claridge, M.F. Dawah, H. A, Wilson, M. R. eds. *Species: The Units of Biodiversity*. New York: Chapman and Hall.

Crandall, K. A., Bininda-Emonds, O. R. P., Mace, G. M. and Wayne, R. (2000) Considering evolutionary processes in conservation biology. *Trends in Ecology and Evolution*, **15:** 290-295.

Creasey, S., Rogers. A., Tyler. P., Young. C. and Gage, J. (1997). The population biology and genetics of the deep-sea spider crab, *Encephaloides armstrongi* Wood-Mason 1891 (Decapoda: Majidae). *Philosophical Transactions of the Royal Society of London, series B*, **352:** 356-376.

Croizat, L. (1964). Space, time, from: the biological synthesis. Published by the author, Caracas.

Crossland, S., Coates, D., Grahame, J. and Mill, P. J. (1993). Use of random amplified polymorphic DNA's (RAPD's) in separating two sibling species of *Littorina. Marine Ecology Progressive Series,* **96:** 301-305.

Crowe, T. (1990). A quantitative analysis of patterns of distribution, species richness and endemism in southern African vertebrates. Pp. 145-160 in Peters G., and R. Hutterer (eds.) Vertebrates in the Tropics. Zoologisches Forschungsinstitus und Museum Koenig, Germany.

Cuesta, J. A. and Schubart, C. D. (1998). Morphological and molecular differentiation between three allopatric populations on the littoral crab *Pachygrapsus transversus*. *Journal of Natural History,* **32:** 1499-1508.

Cumberlidge, N. (1991). *Sudanonautes kagoroensis*, a new species of fresh-water crab (Decapoda: Potamoidea: Potamonautidae) from Nigeria. *Canadian Journal of Zoology,* **69:** 1938-1944.

Cumberlidge, N. and Clarke, P. (1992). A new genus and species of freshwater crab from Cameroon, West Africa (Crustacea, Brachyura, Putamoidea, Potamonautidae). *Bulletin of the British Museum of Natural History (Zoology) London*, **58:** 149-156.

Cumberlidge, N. (1993a). Two new species of *Potamonemus* Cumberlidge and Clark, 1992 (Brachyura: Potamoidea: Potamonautidae) from the rain forests of West Africa. *Journal of Crustacean Biology,* **13:** 571-584.

Cumberlidge, N. (1993b). Rediscription of *Sudanonautes granulatus* (Balss, 1929)(Potamoidea: Potamonautidae) from West Africa. *Journal of Crustacean Biology,* **13:** 804-816.

Cumberlidge, N. (1994). *Louisea,* a new genus of freshwater crab (Brachyura, Potamonoidea, Potamonutidae) for *Globonautes macropus edeaensis* Bott, 1969 from Cameroon. *Proceedings of the Biological Society of Washington*, **107:** 122-131.

Cumberlidge, N. (1995). Redescription of the African freshwater crab *Sudanonautes africanus* (A.Milne Edwards, 1869) (Brachyura: Potamoidea: Potamonautidae). *Journal of Crustacean Biology,* **15:** 588-598.

Cumberlidge, N. (1996). A taxonomic revision of freshwater crabs (Brachyura, Potamoidea, Gecarcinucidae) from the upper Guinea, forest of West Africa. *Crustaceana*, **69:** 681-695.

Cumberlidge, N. (1997). The African and Madagascan fresh-water crabs in the Museum of Natural History, Veinna (Crustacea: Decapoda: Brachyura: Potamoidea). *Annalen des Naturhistorischen Museum in Wein,* **99B:** 571-587.

Cumberlidge, N. (1999a). The Freshwater crabs of West Africa, Family Potamonautidae. *Faune et Flore Tropicales* no 35, IRD, Paris 1-382.

Cumberlidge, N., Von Sternburg, R., Bills, I. R and Martin, H. (1999b). A revision of the genus *Platythelphusa* A. Mile Edwards, 1887 from lake Tanganyika, East Africa (Decapoda: Potamonautidae: Plathelphusidae). *Journal of Natural History*, **33:** 1487-1512.

Cumberlidge, N., Boyko, C. and Harvey A.W. (2002). A new genus and species of freshwater crab (Crustacea, Decapoda, Potamoidea) from northern Madagacar, and a second new species associated with *Pandanus* leaf axils. *Journal of Natural History*, **36:** 65-77.

Cunningham C. W. (1997). Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution,* **14:** 733-740.

Cunningham, C. W., Blackstone, N. W and Buss, L. W. (1992). Evolution of king crab from hermit crab ancestors. *Nature*, **355:** 539-542.

Dai, A. Y. (1999). Fauna Sinica, Crustacea: Decpoda: Parathelphusidae, Potamidae. Science Press, Beijing, China. 1-501, 30pls.

D'Amato, M. E. and Corach, D. (1996). Genetic diversity of populations of the freshwater shrimp *Macrobrachium borelli* (Caridea: Palaemonidae) evaluated by

RAPD analyses. *Journal of Crustacean Biology,* **16:** 650-655.

D'Amato, M. E. and Corach, D. (1997). Population genetic analyses in the fresh water anomura, *Aegla jujuyana*, by RAPD analyses. *Journal of Crustacean Biology,* **17:** 269-274.

Dandy, J.W.T and Ewer, D. W. (1961). The water economy of three species of the amphibious crab, *Potamon*. *Transactions of the Royal Society of South Africa,* **36:** 137-162.

Daniels, S .R., Stewart, B .A. and Gibbons, M. (1998a). Genetic and morphometric variation in the potamonautid river crab *Potamonautes parvispina* (Decapoda; Potamonautidae) from two Western Cape river systems, South Africa. *Journal of Natural History,* **32:** 1245-1258.

Daniels, S. R, Stewart, B. A. and Gibbons, M. (1998b). *Potamonautes granularis* sp. nov (Brachyura, Potamonauitdae) a new cryptic species of river crab from the Olifants River system, South Africa. *Crustaceana,* **71 :** 885-903.

Daniels, S .R., Stewart, B .A. and Gibbons, M. (1999a). Genetic structure amongst populations of *Potamonautes perlatus* (Decapoda: Potamonautidae) from the Olifants River system in the Western Cape, South Africa. *Journal of Zoology London,* **249:** 137-142.

Daniels, S. R., Stewart, B. A. and Gibbons, M. (1999b). Allozyme variation amongst populations of the freshwater crab, *Potamonautes perlatus* in the Berg River system Western Cape. *South African Journal of Zoology,* **34:** 64-68.

Daniels, S. R, Stewart, B. A. and Burmeister, L. (2001a). Geographic patterns of genetic and morphological divergence amongst populations of a river crab (Decapoda, Potamonautidae) with the description of a new species from mountain stream in the Western Cape, South Africa. *Zoologica Scripta,* **30:** 181-197.

Daniels, S. R, Stewart, B. A., Ridgway, T. and Florence, W. (2001b). Carapace dentition patterns, morphometrics and allozyme differentiation amongst two toothed freshwater crab species (*Potamonautes warreni* and *P. unispinus*) (Decapoda: Brachyura: Potamonautidae) from river systems in South Africa. *Journal of Zoology, London*, **255:** 389-404

Daniels, S. R. (2001c). Allometric growth, handedness, and morphological variation in *Potamonautes warreni* (Calman, 1981) (Decapoda: Brachyura, Potamonautidae) with a redescription of the species. *Crustaceana*, **74:** 237-253.

Davies, B., O'Keeffe, J. H. and Snaddon, C. D. (1993). A synthesis of the ecological functioning, conservation and management of South African River ecosystems, Water Research Commission, Cape Town, pp. 1-232.

Deacon, H. J. (1985). An introduction to Fynbos region, time scales and

palaeoenvironments. CSIR Report 75, 1-99.

Department of Environmental Affairs and Tourism. (1997). White Paper on the Conservation and Sustainable Use of South Africa's Biological Diversity. *Government Gazette,* **385:** 1-136.

DeVos, K. M. and Gale, M. D. (1992). The use of random amplified DNA markers in wheat. *Theoretical and Applied Genetics,* **84:** 567-572.

Dowling, T. E. and Secor, C. L. (1997a). The role of hybridization and introgression in the diversification of animals. *Annual Review of Ecology and Systematics,* **28:** 593-619.

Dowling, T. E., Broughton, R. E. and Demarais, B. D. (1997b). Significant role for historical effects in the evolution of reproductive isolation: evidence from patterns of introgression between the cyprinid fishes, *Luxilus cornutus* and *Luxilus chrysocephalus*. *Evolution,* **51:** 1574-1583.

Durand, J. D, Unlu, E., Doadrio, I., Pipoyan, S. and Templeton, A. R. (2000). Origin, radiation, dispersal and allopatric hybridization in the chub *Leuciscus cephalus*. *Proceedings of the Royal Society of London, series B*, **267:** 1687-1697.

Edmands, S. (1999). Heterosis and outbreeding depression in interpopulation crosses spanning a wide range of divergence. *Evolution,* **53:** 1757-1768.

Ellsworth, D. L., Rittenhouse, K. D. and Honeycutt, R. L. (1993). Artifactual variation in evidence for morphological convergence in the morphotypes of *Cochlicopa lubricella* (Gastropoda: Pulmonata: Cochlicopidae). *Journal of Molluscan Studies,* **63:** 379-388.

Farris J.S., Kallersjo M., Kluge, A.G. and Bult, C. (1995). Constructing a significance test for incongruence. *Systematic Biology,* **44:** 570-572.

Felder, D. L. and Staton, J. L. (1994). Genetic differentiation in trans Floridean species complexes of *Sesarma* and *Uca* (Decapoda; Brachyura). *Journal of Crustacean Biology,* **14:** 191-209.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution,* **39:** 783-791.

Ferguson, J. W. H. (1990). Interspecific hybridization in bedbugs supports the Recognition Concept of species. *South African Journal of Science,* **86:** 121-124.

Fetzner, J. (1996). Biochemical systematics and evolution of the crayfish genus *Orconectes* (Decapoda: Cambridae). *Journal of Crustacean Biology,* **16:** 111-141.

Figler, M. H., Twum, M., Finkelstein, J. E. and Peeke, H. (1995). Maternal aggression in red swamp crayfish (*Procambarus clarki*, Girard): The relation between reproductive status and outcome of aggressive encounters with male and female

conspecifics. *Behaviour*, **132:** 107-125.

Finney, W. C. and Abele, L. G. (1981). Allometric variation and sexual maturity on the obligate coral commensal *Trapezia ferruginea* (Decapoda: Xanthidae). *Crustaceana*, **41:** 113-130.

Forbes, A. T., Demetriades, N. T. Benzie, J. A. H. and Balletment, E. (1999). Allozyme frequencies indicating little geographic variation among stock of the giant tiger prawn *Penaeus monodon* in the South-West Indian Ocean. *South African Journal of Marine Science,* **21:** 271-277.

Frost, D. R. and Hillis, D. M. (1990). Species in concept and practice: herpetological applications. *Herpetologica,* **46:** 87-104.

Geller, J. B., Walton, E. D., Grosholz, E. D. and Ruiz, G. M. (1997). Cryptic invasion of the crab *Carcinus* detected by molecular phylogeography. *Molecular Ecology,* **6:** 901-906.

Gherardi, F. and Michelli, R. (1989). Relative growth and population structure of the freshwater crab, *Potamon potamios palaestinensis*, in the Dead Sea area. *Israel Journal of Zoology*, **36:** 250-362

Gibbons, M. J., Abiahy, B. B., Angel, M., Assuncao, C. M. L., Bartsch., I., Best, P., Biseswar, R., Bouillon, J., Bradford-Grieve, J. M., Brach, W., Burreson, E., Cannon,

L., Casanova, J-P., Channing, A., Child, C. A., Compagno, L., Cornelies, P. F. S., Dadon, J. R., David, J. H. M., Day., J., Della Croce, N., Emschermann, P., Erseus, C., Esnal, G., Gibson, R., Griffiths, C. L. Hayward, P. J., Heard, R., Heemstra., P., Hebert, D., Hessler, R., Higgins, R., Hiller, N., Hirano. Y. M., Kensley, B., Kilburn, R., Kornicker, L., Lambshead, J., Manning, R., Marshall, D., Mianzan, H., Monniot, C., Monniot, F., Newman, W., Nielso, C., Patterson, G., Pugh, P., Roeleveld, M., Ross, A., Ryan, P., Ryland., J. S., Samaai, T., Schleyer, M., Schockaert, E., Seapy, R., Shiel, R. Sluys, R., Southward, E. C., Sulaiman, A., Thandar, A., van der Land, J., van der Spoel, S., van Soest, R., Vetter, E., Vinagradov, G., Williams, G. and Wooldridge, T. (1999). The taxonomic richness of South Africa's marine fauna: a crisis at hand. *South African Journal of Science,* **95:** 8–12.

Goldblatt, P. (1978). An analysis of the flora of southern Africa; its characteristics, relationships, and origins. *Annual review of the Missouri Botanical Gardens,* **65:** 369 - 436.

Gomez-Zurita, J., Petitpierre. P. and Juan, C. (2000). Nested cladistic analysis, phylogeography and speciation in the *Timarcha goettingensis* complex (Coleoptera, Chysomelidae). *Molecular Ecology,* **9:** 557-570.

Gordon, I. (1929). A new river-crab of the subgenus *Potamonautes* from Portuguese East Africa. *Annual of the Museum of Natural History*, **3:** 405-412.

Goudet, J. (2000). FSTAT, a program for IBM PC compatibles to calculate Weir and

Cockerham's (1984) estimators of F-statistics (version 2.9.1). Institut de Zoologie et d'Ecologie Animale, Universite de Lausanne, Lausanne.

Gouldstein, P.Z., De Salle, R., Amato, G. and Vogel, A. P. (2000). Conservation genetics at the species boundary. *Conservation Biology,* **14:** 120-131.

Gouws, G. (1999). Genetic and morphological differentiation in potamonautid river crabs (Decapoda: Potamonautidae) from KwaZulu-Natal, South Africa. MSc thesis. University of Stellenbosch. pp 1-240.

Gouws, G, Stewart, B. A and Coke, M. (2000). Evidence for a new species of river crab (Decapoda: Brachyura, Potamonautidae) from the Drakensberg, South Africa. *Journal of Crustacean Biology*, **20:** 743-758.

Gouws, G, Stewart, B. A. and Reavell, P. E. (2001). A new species of freshwater crab (Decapoda, Potamonautidae) from the swamp forests of KwaZulu-Natal, South Africa: biochemical and morphological evidence. *Crustaceana,* **74:** 137-160.

Grandjean, F., Gouin, N., Frelon, M. and Souly-Grosset, C. (1998). Genetic and morphometric systematic studies on the crayfish *Austropotamobius pallipes* (Decapoda: Astacidae). *Journal of Crustacean Biology,* **18:** 549-555.

Grant, W. S., Dempster, Y. L. and Da Silva, F. M. (1988). Use of protein electrophoresis in evolutionary systematics. *Transactions of the Royal Society of*

South Africa, **46:** 295-311.

Guinot, D. (1977). Propositions pour une nouvelle classification des Crustaces decapodes brachyoures. *Comptesreddus hebdomadaires des seances de l'Academie des Science, Paris, series D,* **285:** 1049-1052.

Guinot, D. (1978). Principes d'une classification evolutive des crustaces decapodes brachyoures. *Bulletene biologique de la France et al Belgique, n.s,* **112:** 211-292.

Guinot, D., Jamieson, B. G. M. and Tudge, C. C. (1997). Ultrastructure and relationship of spermatozoa of the freshwater crabs *Potamon fluviatile* and *Potamon ibericum* (Crustacea, Brachyura, Potamidae). *Journal of Zoology London*, **241:** 229-244.

Hadrys, H., Balick, M. and Schierwater, B. (1992). Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Molecular Ecology,* **1:** 55-63.

Halanych, K. M. and Robinson, T. J. (1999). Multiple substitutions affect the phylogenetic utility of cytochrome b and 12S rDNA data: Examining a rapid radiation in leporid (Lagomorpa) evolution. *Journal of Molecular Evolution,* **48:** 369-379.

Hamilton, P. V., Nishimoto, R. T. and Halusky, G. J. (1976). Cheliped laterality in *Callinectes sapidus* (Rathbun). *Biological Bulletin of the Marine Biology Laboratory, Woods Hole*, **150:** 393-794.

Hanner, R. and Fugate, M. (1997). Brachiopod phylogenetics reconstruction from 12 S rRNA sequence data. *Journal of Crustacean Biology,* **17:** 174-183.

Harris, H. (1966). Enzyme polymorphism in man. *Proceedings of the Royal Society of London series B*, **164:** 269-310.

Harrison, A. D. (1978). Freshwater invertebrates (except molluscs). In: Werger, M. J. A. (ed). Biogeography and ecology of Southern Africa. Volume 2. Dr. W. Junk Publishers, The Hague. Pp. 1139-1152.

Harrison, M. K. and Crespi, B. J. (1999). Phylogenetics of Cancer crabs (Crustacea: Decapoda: Brachyura). *Molecular Phylogenetics and Evolution,* **12:** 186-199.

Hartl, D. L and Clark, A. G. (1989). Principals of population genetics. 2nd edition. Sinauer Associated Publishers, Sunderland, Massachusetts, U.S.A.

Hartnoll, R. G. (1982). Growth. In, L. G. Abele (ed), The Biology of Crustacea, 2: 111-196. London: Academic Press.

Hasegawa, M., Kishino, K. and Yano, T. (1985). Dating the human ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution,* **22:** 106-174.

Hebert, C., Danzman, R. G., Jones, M. W. and. Bernatchez, L. (2000). Hydrography and population genetic structure in brook charr (*Salvelinus fontinalis*, Mitchill) from eastern

Canada. *Molecular Ecology,* **9:** 971-982.

Hedgecock, D., Tracy, M. L. and Nelson, K. (1982). 6 Genetics., *The biology of Crustacea, Volume II. Embryology, Morphology and Genetics*. In: L.G. Abele (eds), New York: Academic Press. pp 283-403.

Hewitt, G. M. (1996). Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society,* **58:** 247-276.

Hewitt, G. M. (2000). The genetic legacy of the quaternary ice ages. *Nature*, **405:** 907-913.

Hill, M. P. and O'Keeffe, J. H. (1992). Some aspects of the ecology of the freshwater crab (*Potamonautes perlatus*) in the upper reaches of the Buffalo River, Eastern Cape province, South Africa. S*outh African Journal of Aquatic Sciences,* **18:** 42-50.

Hillis, D. M. (1987). Molecular versus morphological approaches to systematics. *Annual Review of Ecology and Systematics,* **18:** 23-42.

Hillis, D. M., Moritz, C. and Mable, B. K. (1996). Molecular Systematics, 2nd edition, MA Sinauer Associates, Sutherland, U.S. A.

Holthuis, L. B. (1964). Forty-seven genera of Decapoda Crustacea placed on the official list. *Bulletin of Zoological Nomenclature,* **21:** 336-351.

Huelsenbeck, J. P., Bull, J. J. and Cunningham, W. (1997). Combining data in phylogenetic analysis. *Trends in Ecology and Evolution*, **11:** 152 - 157.

Hubby, J. L. and Lewontin, R. C. (1966). A molecular approach to the study of genic heterozygosity in natural populations. I. The number of alleles at diferent loci in *Drosophila pseudoobscura*. *Genetics*, **54:** 577-594.

Hughes, J. M., Bunn, S. E., Kingston, D. M. and Hurwood, D. A. (1995). Genetic differentiation and dispersal among populations of *Paratya australiensis* (Atyidae) in rainforest streams in southeast Australia. *Journal of the North American Benthological Society*, **14:** 158-173.

Hughes, J. M., Bunn, S. E., Choy, S. Hurwood, D. A. and Pearson, R. G. (1996). Genetic differentiation among populations of *Caridina zebra* (Atyidae) in tropical rainforest streams, northern Australia. *Freshwater Biology,* **36:** 289-296.

Hurwood, D. A and Hughes. J. M. (2001). Nested clade analysis of the freshwater shrimp, *Caridina zebra* (Decapoda: Atidae), from North-Eastern Australia. *Molecular Ecology*, **10:** 113-125.

Innes, M. A., Gelfa, D. H., Sninsky, J. J and White, T. (1990). PCR protocols. Academics, San Diego.

IUCN Red List Categories. (2000). IUCN Species Survival Commission,

Switzerland.

James, F. and Mc Culloch, R. (1990). Multivariate analyses in ecology and systematics: Panacea or Pandora's box? *Annual Review of Ecology and Systematics,* **21:** 129-166

Jamieson, B. G. M. (1990). The ultrastructure of the spermatozoa of *Peralomera lateralis* (Gray) (Crustacea, Brachyura, Dromiacea) and its phylogenetic significance. *Invertebrate Reproduction and Development,* **17:** 39-45.

Jubb, R. A. and Farquharson, F. L. (1965). The freshwater fishes of the Orange River drainage basin. *South African Journal of Science,* **61:** 118-125.

Karl, S. A. and Avise, J. C. (1992). Balancing selection at allozyme loci in oysters: Implications from nuclear RFLP' s. *Science,* **256:**100-102.

Karl, S. A. and Bowen, B.W. (1999). Evolutionary significant units versus geopolitical taxonomy: molecular systematics of an endangered sea turtle (genus *Chelonia*). *Conservation Biology,* **13:** 990-999.

Katoh, M. and Foltz, D. W. (1994). Genetic subdivision and morphological variation in a freshwater snail species complex formerly referred to as *Viviparus georgianus* (Lea). *Biological Journal of the Linnean Society,* **53:** 73-90.

Kensley, B. (1981). On the zoogeography of Southern African decapod crustacea, with a distributional checklist of species. *Smithsonian Contributions to Zoology*, **338:** 1-64.

Kim, W. and Abele, L .G. (1990). Molecular phylogeny of selected decapod Crustaceans based on 18S rRNA nucleotide sequences. *Journal of Crustacean Biology,* **10:** 1-13.

Kluge, A. J. (1989). A concern for evidence and a phylogenetic hypothesis of relationships among *Epicrates* (Boidea, Serpentes). *Systematic Zoology,* **38:** 7-25.

Knowlton, N. (1986). Cryptic and sibling species amongst the decapod Crustacea. *Journal of Crustacean Biology,* **6:** 356-363.

Knowlton, N. (1992). The systematic importance of color and color patterns. Evidence for complexes of sibling species of snapping shrimp (Caridea: Alpheidea: *Alpheus*) from the Caribbean and Pacific coast of Panama. *Proceedings of the San Diego Society of Natural History,* **18:** 1-5.

Knowlton, N. (1993). Sibling species in the sea. *Annual Review of Ecology and Systematics,* **24:** 189-216.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F. X. and Wilson, A. C. (1989). Dynamics of mitochodrial DNA evolution in animals:

Aplification and sequencing with conserved primers. *Proceedings of the National Academy of Science, USA,* **86:** 6196-6200.

Krauss, F. (1843). Die Sudafrikanischen Crustaceen - Eine Zusammenstellung aller bekkannten Malacostraca. E. Schweizerbart, Stuttgard, Germany. Pp. 1-68.

Langor, D.W. and Sperling, F. A. H. (1997). Mitochondrial DNA sequence divergence in weevils of the *Pissodes strobi* species complex (Coleoptera: Curculioinidae). *Insect Molecular Biology*, **6:** 255-265.

Lee, M. S. Y. (2001). Uninformative characters and apparent conflict between molecules and morphology. *Molecular Biology and Evolution,* **18:** 676-680.

Levene, H. (1949). On a matching problem arising in genetics. *Annals of Mathematical Statistics,* **20:** 91-94.

Lewontin, R. C. and Hubby, J. L. (1966). A molecular approach to the study of heterozygosity in natural populations. II Amount of variation and degree of heterozygosity in natural populations of *Drosophila pseudoabscura*. *Genetics*, **54:** 596-609.

Liu, H and Li, C. (2000). Reproduction in the fresh-water crab *Candidiopotamon rathbunae* (Brachyura: Potamidae) in Taiwan. *Journal of Crustacean Biology*, **20:** 8-99.

Li, W. H. and Graur, D. (1991). Fundamentals of molecular evolution. 284 pp. Sunderland, MA: Sinauer Associates Inc.

Lynch, M. and Jarrell, P. E. (1993). A method of calibrating molecular clocks and its application to animal mitochondrial DNA. *Genetics,* **135:** 1197-1208.

Lynch, M. and Mulligan, B.G. (1994). Analyses of population genetic structure with RAPD markers. *Molecular Ecology,* **3:** 91-99.

Maddison, W. P. and Maddison, D. P. (1992). MacClade. Analysis of phylogeny and character evolution. Sinauer Associates Inc., Sunderland, Massachusetts.

Maltagliati, F., Camilli, L., Biage, F. and Abbiati, M. (1998). Genetic structure of Norway lobster, *Nephrops norvegicus* (L) (Crustacea: Nephropidae) from the Mediterranean Sea. *Scientia Marina,* **62:** 91-99.

Mardulyn, P., Milinkovitch, M. C. and Pasteels, J. M. (1997). Phylogenetic analysis of DNA and allozyme data suggest that *Gonioctena* leaf beetles (Coleoptera: Chrysomelidae) experienced convergent evolution in their history of host-plant family shifts. *Systematic Biology*, **46:** 722-747.

Markert, C. L. and Faulhaber, I. (1965). Lactate dehydrogenase isozyme patterns of fish. *Journal of Experimental Zoology,* **159:** 319-332.

Matthee, C., Burzlaff, J. D., Taylor, J. and Davis, S. K. (2001). Mining the Mammalian genome for Artiodactyl systematics. *Systematic Biology,* **50:** 367-390.

Mafield, S. (1993). Aspects of the distribution, population structure and feeding biology of the freshwater crab, *Potamonautes perlatus* (Milne Edwards) (Decapoda: Potamonidae) in the Liesbeen River, Western Cape, South Africa. B.Sc (Hons) thesis, University of Cape Town.

Mayr, E. (1964). Animal species and evolution. Harvard University Press, Cambridge, Massachusetts.

Mayr, E. (1970) Populations, Species and Evolution. Harvard University Press, Cambridge, Massachusetts.

McClure, M. and Wicksten, M. K. (1997). Morphological variation of species of the Edwardsii group of *Alpheus* in the northern Gulf of Mexico and Northwestern Atlantic (Decapoda; Caridea: Alpheidae). *Journal of Crustacean Biology,* **17:** 480-487.

McDade, L. (1990). Hybrids and phylogenetic systematics I. Patterns of character expression in hybrids and their implications for cladistic analysis. *Evolution*, **44:** 1685-1700.

McDade, L. (1992). Hybrids and phylogenetic systematics II. The impact of hybrids on caldistic analysis. *Evolution*, **46:** 1329-1346.

McMillen-Jackson, A. M., Bert, T. M. and Steele, P. (1994). Population genetics of the blue crab *Callinectes sapidus*: modest population structure in a background of high gene flow. *Marine Biology,* **118:** 53-65.

Merkouris, S. E. and Seeb, L.W. (1998). Low levels of genetic diversity in highly exploited populations of Alaskan Tanner crabs, *Chionoecetes bairdi* and the Alaskan and Atlantic snow crabs *C. opilio*. *Fisheries Bulletin*, **96:** 525-537.

Meyer, A. (1994). Shortcomings of the cytochrome b gene a molecular marker. *Trends in Ecology and Evolution,* **9:** 278-280.

Mickevich, M. F and Mitter, C. (1983). Evolutionary patterns in allozyme data: a systematic approach. In: Platnick, N. I and Funk, V. A. (eds.). Advances in Cladistics, Volume 2. Proceedings of the Second Meeting of the Willi Henning Society. Columbia University Press, New York. Pp. 169-179.

Milne-Edwards, A. (1868). Description de quelques Crustaces nouvequx. *Nouvelles Archives du Museum d'Naturelle,* **4:** 69-92.

Milne-Edwards, H. (1837). *Histroire Naturelle des Crustaces Paris,* **2:** 1-532.

Milne-Edwards, H. (1853). Observations sur les affinites zoologiques et al classification naturelle des Crustaces. *Annales des Sciences Naturelles, Zoologie, Paris,* **20:** 163-228.

Miyamoto, M. M. and Fitch ,W. M. (1995). Testing species phylogenies and phylogenetic methods with congruence. *Systematic Biology,* **44:** 64-67.

Monod, T. (1977). Sur quelques crustaces decapodes africains (Sahel, Soudan). *Bulliten Museum Natural History Series A- Zoology*, **349:** 1201-1236.

Moor, A. E. (1988). Plant distribution and the evolution of the major river systems in southern Africa. *South African Journal of Geology,* **91:** 346-349.

Moore, W. S. and Price, J. T. (1993). Nature of selection in the northern flicker hybrid zone and its implication for speciation theory. In: Harrison, R. G. (ed.). Hybrid Zones and the Evolutionary Process. Oxford University Press, Oxford. Pp. 196-225.

Moritz, C. (1994). Defining 'Evolutionary Significant Units" for conservation. *Trends in Ecology and Evolution*, **9:** 373-375.

Mullis, K.B., Faloona, F., Scharf, S., Saiki, R., Horn, G. and Erlich., H. (1986). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Symposium, Quantinary Biology*, **51:** 263-273.

Murphy, R. W and Doyle, K. D. (1998). Phylophenetics: frequencies and polymorphic characters in genealogical estimations. *Systematic Biology,* **47:** 737-761.

Myers, N. (1990). The biodiversity challenge: expanded hot-spot analysis. *The*

Environmentalist, **10:** 243-255.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics,* **89:** 583-590.

Nei, M. (1977). F- statistics and analysis of gene diversity in sudbivided populations. *Annals of Human Genetics*, **41:** 225-233.

Nei, M., Roychoudhury, A. K. (1974). Sampling variance of heterozygosity and genetic distance. *Genetics,* **76:** 379-390.

Nelson, K. and Hedgecock, D. (1980). Enzyme polymorphism and adaptive strategy in the Decapod Crustacea. *American Naturalist,* **116:** 238-280.

Ng, P. K. L. and Tan, L.W. H. (1985). "Right handedness" in the heterochelous calappoid and xanthid crabs, suggestion for functional advantages. *Crustaceana*, **49:** 98-100.

Ng, P. K. L. and Ng, P. (1987). The freshwater crabs in Pulau Langkawi, Peninsular Malysia. *Malaysian Journal of Science*, **9:** 1-15.

Ng, P. K. L. (1988). The freshwater crabs of Peninsular Malysio and Singapore. Department of Zoology, National Univeristy of Singapore, Shiglee Press, Singapore, pp i-viii, 1-156

Ng, P. K.L. and Takeda, M. (1994). *Skelosophusa* (Crustacea: Decapoda: Brachyura) a new genus of potamonautid freshwater crabs from Madagascar, with the description of two new species. *Bulletin of the National Science Museum, Tokyo, Series A,* **20:** 161-172.

Ng, P. K. L. Stevcic, Z and Pretzman G. (1995a). Arevision of the family Deckeniidae, 1897 (Crustacea: Decapoda: Brachyura: Potamonautidae), with decription of a new genus (Gecarcinucidae: Gecarcinucoidea) from the Seychelles, Indian Ocean. *Journal of Natural History,* **29:** 581-600.

Ng, P. K. L. and Rodriguez, G. (1995b). Freshwater crabs as poor zoogeographic indicators: A critique of Banarescu (1990). *Crustaceana,* **68:** 636-645.

Ng, P. K. L. and Sket, B. (1996). The freshwater crab fauna (Crustacea: Brachyura) of the Philippines IV. On a collection of Parathelphusidae from Bohol. *Proceedings of the Biological Society of Washington,* **109:** 695-706.

O'Keeffe, J.H., Davies, B.R., King, J.M. and Skelton, P.H. (1989). The conservation status of South African Rivers. In: Biotic diversity in South Africa. Concepts and conservation. B.J. Huntley (ed). Oxford University Press, Cape Town. pp 266-289.

Ortmann, A. E. (1897). Carcinologische Studien. *Zoologische Jahrbucher fur Systematik,* **10:** 258-372.

Ortmann, A. E. (1896). Das system der Decapoden-Krebse. *Zoologische Jahrbucher furn Systematik,* **9:** 409-453.

Ortmann, A. E. (1902). The geographic distribution of freshwater decapods and its bearing upon ancient geography. *Proceedings of the American Philosophical Society,* **41:** 267-400.

Overton, J. L., Macintosh, D. J. and Thorpe, R. S. (1997). Multivariate analysis of the mud crab S*cylla serrata* (Brachyura: Portunidae) from four localities from Southeast Asia. *Marine Biology,* **128:** 55-65.

Palumbi, S. R. (1996). Nucleic acid II. The polymerase chain reaction. In "Molecular Systematics" Hillis, D, Moritz, C and Mable, B. K. pp205-248. Sinauer, Sunderland, MA.

Palumbi, S. R., Martin, A. Romano, W., McMillan, O., Stice, L. and Grabowski, G. (1991). The simple fools guide to PCR, version 2.0. Distributed by authors, University of Hawaii, Honolulu.

Parker, P., Snow, G., Schug, M. D., Booton., G., C. and Fuerst, P. A. (1998). What molecules can tell us about populations: choosing and using a molecular marker. *Ecology*, **79:** 361-382.

Passamonti, A., Mantovani, B., Scali, V. and Froglia, C. (1997). Allozyme

characterization of the Scottish and Aegean Populations of *Nephrops norvegicus*. *Journal of Marine Biological Association,* **77:** 727-735.

Paterson, H. E. H. (1985) The recognition concept of species. *In:* Vrba, E. S. (ed.). *Species and Speciation. Transvaal Museum Monograph No. 4.* Transvaal Museum, Pretoria. Pp. 21-29.

Perez, M. L., Valverde, J. R., Batuecas, B., Amat, F., Marco, R. and Garesse, R. (1994). Specistion in the *Artemia* genus: mitochondrial DNA analysis of bisexual and parthenogenetic brine shrimps. *Journal of Molecular Evolution*, **38:** 156-168.

Petit, C., Bretagnolle, F. and Felber, F. (1999). Evolutionary consequences of diploid-polyploid hybrid zones in wild species. *Trends in Ecology and Evolution*, **14**: 306-311.

Piel, W. H and Nutt, K. J. (2000). One species or several ? Discordant patterns of geographic variation between allozymes and mtDNA sequences among spiders in the genus *Metepeira. Molecular Phylogeny and Evolution*, **15:** 414 - 418.

Posada, D and Crandall, K. A. (1998). MODELTEST: Testing the model of DNA substitution. *Bioinformatics,* **14:** 817-817.

Posada, D and Crandall, K. A. (2001). Selecting the best-fit model of nucleotide substitution. *Systematic Biology,* **50:** 580-601.

Purves, M. G., Kruuk, H. and Nel, J. A. J. (1991). Crabs *Potamonautes perlatus* in the diet of Otter *Anoyx capensis* and the water mongoose *Atilax paludinosus* in a freshwater habitat in South Africa. *International Journal of Mammalian Biology*. **59:** 332-341.

Rathbun, M. J. (1904). Les crabes d'eau douce (Potamonidae). *Nouvelles Archives du Museum d'Histoire Naturelle, Paris*, **4:** 225-312.

Rathbun, M. J. (1905). Les crabes d'eau douce (Potamonidae). *Nouvelles Archives du Museum d' Histoire naturelle, Paris,* **7:** 159-322.

Rathbun, M. J. (1906). Les crabes d'eau douce (Potamonidae). *Nouvelles Archives du Museum d' Histoire naturelle, Paris,* **8:** 106-124.

Rathbun, M. J. (1909). Collections recueilles par M. Maurice de Rothschild dans L' Afrique orientale Anglaise. Crabes d'eau douce nouveaux. *Bulletin du Museum d'Histoire Naturell,* **109:** 101-105.

Rathbun, M. J. (1921). The Brachyuran crabs collected by the American Museum Congo expedition, 1909-1915. *American Museum of Natural History Bulletin,* **43:** 400-417.

Raubenheimer, C. D. (1986). Aspects of the biology of the freshwater crab, *Potamonautes sidneyi*, with particular reference to general seasonality and female aggression. M.Sc. thesis, Department of Biological Sciences, University of Natal,

Durban.

Rhymer, J. H and Simberloff, D. (1996). Extinction by hybridization and introgression. *Annual Review of Ecology and Systematics*, **27:** 83-109.

Rice, A. L. (1980). Crab zoel morphology and its bearing on the classification of the Brachyura. *Transactions of the Zoological Society of London,* **35:** 271-424.

Richardson, B. J, Baverstock, P. R and Adams, M. (1986). Allozyme electrophoresis: A handbook for animal systematics and population studies. Academic Press, Sydney.

Riddle, B. (1996). The molecular phylogeographic bridge between deep and shallow history in continental biotas. *Trends in Ecology and Evolution,* **11:** 207-211.

Ridgeway, G., Sherburne, S. W and Lewis, R. D. (1970). Polymorphism in the esterase of Atlantic herring. *Transactions of the American Fisheries Society,* **99:** 147-151.

Ricker, W. E. (1973). Linear regression in fishery research. *Journal of Fisheries Research Canada*, **30:** 409-434.

Ricker, W. E. (1975). A note concerning Professor Jolicoeur's comments. *Journal of Fisheries Research Canada*, **32:** 1494-1498.

Rodgers, J. S. (1972). Measures of genetic similarity and genetic distance. *Studies in Genetics VII. University of Texas Publications,* **7213:** 145-153.

Rodgriguez, F. J., Oliver, J., Martin, L., and Medina, J. R. (1990). The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology*, **142:** 485-501.

Rodriguez, G. and Campos, M. (1989). Cladistic relationships of the freshwater crabs of the tribe Strengerianini (Decapoda: Pseudothelphusidae) from the Northern Andes, with comments on their biogeography and description of new species. *Journal of Crustacean Biology,* **9:** 141-156.

Rodriguez, G. (1992a). The Freshwater crabs of America; Family Trichodactylidae and supplement to the family Pseudothelphusidae. Ostrom edition, *Fauna Tropicale,* **31:** 1-189.

Rodriguez, G. and Pereira, G. (1992b). New species, cladistic relationships, and biogeography of the genus *Fredius* (Decapoda: Brachyura: Pseudothelphusidae) from South America. *Journal of Crustacean Biology,* **12:** 298-311.

Roe, K. J and Lydeard, C. (1998). Species delineation and the identification of evolutionarily significant units; lessons from the freshwater mussel genus *Potamilus* (Bivalvai: Unionidae). *Journal of Shellfish Research*, **17:** 1359-1363.

Rohlf, F. J. and Marcus, L. F. (1993). A revolution in morphometrics. *Trends in Ecology and Evolution,* **8:** 129-132.

Rosa, D. (1918). Ologenesi, Nuova teoria. Dell' origine e della distribuzione geografica dei viventi: Firenze.

Russell, J. and Rollingson, D. (1996). An evaluation of random amplified polymorphic DNA (RAPD) for the identification and phylogeny of freshwater snails of the genus *Bulinus* (Gastropoda: Planorbidea). *Journal of Molluscan Studies,* **62:** 165-176.

Ryder, O. A. (1986). Species conservation and systematics: the dilemma of subspecies. *Trends in Ecology and Evolution,* **1:** 9-10.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. and Arnheim, N. (1985). Enzymatic amplification of B - globin genomic sequences and restriction site analysis for diagnosis of sickles cell anemia. *Science*, **230:** 1350-1354

Sang, T. and Zhong, Y. (2000). Testing hybridization hypothesis based on incongruent gene trees. *Systematic Biology,* **49:** 422-434.

Sbordoni, M.C., Mattoccia, M., La Rosa,P., De Matthaeis, E and Sbordoni, V. (1990). Secondary sympatry occurrence of sibling species of subterranean shrimps in the Karst. *International Journal of Speleology,* **19:** 9-27.

Schneider, S., Kueffer, J., Roessli, D. and Exoffier, L. (1997). ARLEQUIN: Exploratory molecular population genetics software, version 1. University of Geneva.

Schon, I., Butlin, R., Griffiths, H. and Martins, K. (1998). Slow molecular evolution in an ancient asexual ostracod. *Proceedings of the Royal Society of London, series B*, **265:** 235-242.

Schubart, C. D, Neigel, J. E and Felder, D. L. (2000). Molecular phylogeny of mud crabs (Brachyura: Panopeidae) form the Northwest Atlantic and the role of morphological stasis and convergence. *Marine Biology,* **137:** 11-18.

Schubart, C. D., Diesel, R. and Hedges, S. B. (1998b). Rapid evolution to terrestrial life in Jamaican crabs. *Nature,* **393:** 363-365.

Schubart, C.D., Reimer, J. and Diesel, R. (1998a). Morphological and molecular evidence for a new endemic freshwater crab, *Sesarma ayatum* sp. n., (Grapsidae, Sesarminae) from eastern Jamaica. *Zoologica Scripta*, **27:** 373-380.

Schwenk, K., Posada, D. and Herbert, P. D. N. (2000). Molecular systematics of the European *Hyalodaphnia*: the role of contemporary hybridization in ancient species. *Proceedings of the Royal Society of London, series B*, **267:** 1833-1842.

Seeb, L. W, Seeb, J. E. and Polovina, J, J. (1990). Genetic variation in highly exploited spiny lobster *Panulirus marginatus* populations from the Hawaiian archipelago.

Fisheries Bulletin, **88:** 713-718.

Shaw, C. R. and Prasad, R. (1970). Starch gel electrophoresis of enzymes - a compilation of recipes. *Biochemical Genetics,* **4:** 297-320.

Shimodaira, H. and Hasegawa, M. (1999). Multiple comparisons of log-likelihoods with applications to phylogenetic inferences. *Molecular Biology and Evolution,* **16:** 114 -116.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. and Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America,* **87:** 651-701.

Simpson, G. G. (1961). Principles of Animal Taxonomy. Columbia University Press, New York.

Sites, J. W. Jr. and Crandall, K. A. (1997). Testing species boundaries in biodiversity studies. *Conservation Biology,* **11:** 1289-1297.

Skelton, P. H. (1994). Diversity and distribution of freshwater fishes in East and Southern Africa. *Annals of the Museum of African Center for Zoology,* **275:** 95-131.

Slatkin, M. and Voelm, L. (1991). $F_{(ST)}$ in a hierarchical island model. *Genetics*, **127:**

627-629

Slatkin, M. (1995). A measure of population subdivision based on microsatellite allele frequencies. *Genetics,* **139:** 457-462.

Smith, G. R. (1992). Introgression in fishes: significance for paleontology, cladistics, and evolutionary rates. *Systematic Biology,* **41:** 41-57.

Somers, M. J. and Nel. J. A. J. (1998). Dominance and population structure of freshwater crabs (*Potamonautes perlatus* Milne Edwards). *South African Journal of Zoology*, **33:** 31-36.

Stasoft, Inc. (1996). STATISTICA for Windows. Tulsa, Oklahoma, U. S. A.

Staton, J. L. and Felder, D. L. (1995). Genetic variation in populations of the ghost shrimp genus *Callichirus* (Crustacea: Decapoda: Thalassinoidea) in the western Atlantic and Gulf of Mexico. *Bulletin of Marine Science,* **56:** 523-536.

Stein, R. A. (1976). Sexual dimorphism in crayfish chelea: functional significance linked to reproductive activities. *Canadian Journal of Zoology*, **54:** 220-227.

Stephan, W. and Langley, C. H. (1992). Evolutionary consequences of DNA mismatch inhibited repair opportunity. *Genetics,* **132:** 567-574.

Stevens, P. M. (1990). A genetic analyses of the pea crabs (Decapoda: Pinnotheridea) of New Zealand. 1. Patterns of spatial and host-associated genetic structure in *Pinnotheres novaezelanidiae* Filhol. *Journal of Experimental Marine Biology and Ecology,* **141:** 195-212.

Stewart, B. A. (1992a). Morphological and genetic differentiation between allopatric populations of the freshwater amphipod. *Journal of Zoology, London,* **228:** 287-305.

Stewart, B. A. and Griffiths, C. L. (1992b). Four new species of the genus *Paramelita* (Amphipoda, Crangonyctoidea) from South Africa. *Annals of the South African Museum*, **101:** 139 -158.

Stewart, B. A. and Griffiths, C. L. (1992c). Four new species of the genus *Paramelita* (Amphipoda, Crangonyctoidea) from South Africa. *Annals of the South African Museum*, **104:** 101-185.

Stewart, B. A. and Griffiths, C. L. (1992d). Further new species within the freshwater amphipod genus *Paramelita* from South Africa. *Journal of Natural History*, **26:** 489-506.

Stewart, B. A., Coke, M. and Cook, P. A. (1995). *Potamonautes dentatus*, new species, a freshwater-crab (Brachyura: Potamoidea: Potamonautidae) from KwaZulu-Natal, South Africa. *Journal of Crustacean Biology,* **15:** 558-568.

Stewart, B. A. (1997a). Biochemical and morphological evidence for a new species of river crab *Potamonautes parvispina* sp. nov (Brachyura, Potamonautidae). *Crustaceana* **70:** 737-753.

Stewart, B. A. (1997b). Morphological and genetic differentiation between populations of river crabs (Decapoda: Potamonautidae) from the Western Cape, South Africa, with a taxonomic re-examination of *Gecarcinautes brinki*. *Zoological Journal of the Linnean Society,* **119:** 1-21.

Stewart, B. A and Cook, P. A. (1998). Identification of a new species of river crab (Decapoda: Brachyura: Potamonautidae) from South Africa using morphology and genetic data. *Journal of Crustacean Biology,* **18:** 48-61.

Stott, W., Fergurson, M. and Tallman, R. F. (1992). Genetic population structure of American plaice (*Hippoglossaides platessoides*) from the Gulf of St Lwarence, Canada. *Canadian Journal of Fisheries and Aquatic Sciences*, **49:** 2538-2545.

Strumbauer, C., Levinton, J. S. and Christy, J. (1996). Molecular phylogeny analysis of fiddler crabs: test of the hypothesis of increasing behavioral complexity in evolution. *Proceedings of the National Academy of Science USA*, **93:** 10855-10857.

Stuckenberg, R. (1962). The distribution of the montane palaeogenic elements in the South African invertebrate fauna. *Annals of the Cape Province Museum,* **II:** 190-205.

Stuckenberg, R. (1969). Effective temperature as an ecological factor in southern Africa. *Zoologica Africana,* **4:** 145-197.

Sundberg, P. and Anderson, S. (1995). Random amplified polymorphic DNA (RAPD) and intraspecific variation in *Oerstedia dorsalis* (Hoplonemertea, Nemertea). *Journal of Marine Biology Associates,* **27:** 483-490.

Suzuki, H. and Okano T. (2000). A new freshwater crab of the genus *Geothelphusa* Stimpson, 1858 (Crustacea: Decapoda: Brachyura: Potamidae) from Yakushima Island, southern Kyushu, Japan. *Proceedings of the Biological Society of Washington,* **113:** 30-38.

Swofford, D. L. and Selander, R. B. (1981). BIOSYS-1: a computer program for the analyses of allelic variation in population genetics and biochemical systematics. release 1.7. David L. Swofford, Illinois Natural History survey.

Swofford, D. L, Olsen, G. J, Waddell, P. J and Hillis, D. M. (1996). Phylogeny reconstruction. In Hillis, D. M, Moritz, C and Mable, BK. Eds. Molecular systematics. 2nd edition. Sunderland, Massachusetts; Sinauer Associates, 407-514.

Swofford, D. L., (2001). Phylogenetic Analysis Using Parsimony and other Methods (PAUP*), 4 version beta 8. Sinauer, Sunderland, MA.

Tam, Y. K. and Kornfield, I. (1998). Phylogenetic relationships of clawed lobsters

enera (Decapoda: Nephropidae) based on mitochondrial 16 S rRNA gene sequences. *Journal of Crustacean Biology*, **18:** 138-146.

Tam, Y. K. and Chu, K. H. (1993). Electrophoretic study on the phylogenetic relationships of some species of *Penaeus* and *Metapenaeus* (Decapoda: Penaeidae) from the South China Sea. *Journal of Crustacean Biology,* **13:** 697-705.

Taylor, D. J, Finston, T. L. and Hebert, P. D. N. (1998). Biogeography of a widespread freshwater crustacean: pseudocongruence and cryptic endemism in the North American *Daphnia laevis* complex. *Evolution*, **52:** 1648-1670.

Taylor, D. J., Hebert, P. D. N. and Colbourne, J. K. (1996). Phylogenetics and evolution of the *Daphnia longispina* group (Crustacea) based on 12S rDNA sequence and allozyme variation. *Molecular Phylogenetics and Evolution,* **5:** 495-510.

Templeton, A. R. (1981). Mechanisms of speciation – a population genetic approach. *Annual Review of Ecology and Systematics.* **12:** 23-48.

Templeton, A. R. (1980). Modes of speciation and inferences based on genetic distances. *Evolution,* **34:** 719-729.

Templeton, A. R. (1989). The meaning of species and speciation: a genetic perspective. Page 3-27, In. D Otte and J A Endler, editors. Speciation and its consequences. Sinauer Associates, Sunderland, Massachusetts.

Thompson, A P. (1996). A genetic perspective on the specific status of the western rock lobster along the coast of western Australia - *Panulirus cygnus* George, 1962 or *P. longipes* A Milne - Edwards 1868? *Journal of the Royal Society of Western Australia,* **79:** 207-210.

Thompson, J. D, Higgens, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acid Research,* **22:** 4673-4680.

Thorpe, J. P. (1982). The molecular clock hypothesis: biochemical evolution, genetic differentiation and systematic. *Annual Review of Ecology Systematics,* **13:** 139-168.

Thorpe, J. P. and Solé-Cava, A. M. (1994). The use of allozyme electrophoresis in invertebrate systematics. *Zoologica Scripta,* **23:** 3-18.

Trewick, S. A., Wallis, G.P. and Morgan-Richards, M. (2000). Phylogeographic pattern correlates with Pliocene mountain building in the alpine scree weta (Orthoptera, Anostostomatidae). *Molecular Ecology*, **9:** 657-666.

Tyson, P. D. (1986). Climatic change and variability in Southern Africa. Oxford University Press, Southern Africa.

Van de Zande, L. and Bijlsma, R. (1995). Limitations of the RAPD technique in phylogeny reconstruction in *Drosophila*. *Journal of Evolutionary Biology*, **8:** 645-656.

Von Sternberg, R. and Cumberlidge, N. (1999b). A cladistic analysis of *Platythelphysa* A. Milne-Edwards, 1887, from Lake Tanganyika, East Africa (Decapoda: Potamoidea: Platythelphusidae) with comments on the phylogenetic position of the group. *Journal of Natural History,* **33:** 493-511.

Von Sternberg, R., Cumberlidge, N. and Rodriguez, G. (1999a). On the marine sister groups of the freshwater crabs (Crustacea: Decapoda: Brachyura). *Journal of Zoology and Systematic and Evolutionary Research,* **37:** 19-38.

Waples, R. S. (1987). A multispecies approach to the analysis of gene flow in marine shore fishes. *Evolution,* **41:** 385-400.

Waples, R. S. (1991). Pacific salmon, *Onycorhynchus* spp., and the definition of species under the endangered species act. *Marine and Fisheries Review*, **53:** 11-12.

Waples, R. S. (1995). Evolutionary significant units and the conservation of biological diversity under the endangered species act. Page 8-27 in J. L Nielson, editor. Evolution and the aquatic ecosystems: defining unique units in the population conservation. Symposium 17. American Fisheries Society, Bethesda, Maryland.

Wayne, R. K. (1992). On the use of morphological and molecular characters to

investigate species status. *Conservation Biology*, **6:** 590-592.

Weir, B. S. and Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution*, **38:** 1358-1370.

Welsh, J. and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic acid Research,* **18:** 7213-7218.

Werger, M. J. A. (1978). Biogeography and ecology of Southern Africa. W. Junk, The Hague, Netherlands.

Whelman, S., Lio, P. and Golgman, N. (2001). Molecular phylogenetics; state-of-the-art methods for looking into the past. *Trends in Genetics*, **17:** 262 - 272.

Whitt, G. S. (1970). Developmental genetics of the lactate dehydrogenase isozymes of fish. *Journal of Experimental Zoology*, **175:** 1-35.

Wiens, J. J. (1995). Polymorphic characters in phylogenetic systematics. *Systematic Biology,* **44:** 428-500.

Wiens, J J. (2000a). Reconstructing phylogenies from allozyme data: comparing method performance with congruence. *Biological Journal of the Linnean Society,* **70:** 613-632.

Wiens, J. J. (2000b). Phylogenetic analysis of morphological data. Smithsonian

institutional Press, Washington, U.S.A.

Wild, H. (1968). Phytogeography in south central Africa. *Kirkia,* **6:** 197-222

Wiley, E. O. (1978). The evolutionary species concept reconsidered. *Systematic Zoology,* **27:** 17-26.

Williams, J., Kubelik, A. R., Livak, K. J., Rafaliski, J. A. and Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful genetic markers. *Nucleic acid research,* **18:** 6531-6535.

Wishart, M. J. and Hughes, J. M. (2001). Allozyme variation between conspecific populations of the net-winged midge, *Elporia barnardi* (Diptera: Blephariceridae), in mountain streams of the southwest Cape, South Africa. *Freshwater Biology*, **46:** 479-490.

Woodruff, D. S. (1989). Genetic anomalies associated with *Cerion* hybrid zones: the origin and maintenance of new electromorphic variants called hybrizymes. *Biological Journal of the Linnean Society,* **36:** 281-294.

Woolschot, L., Hughes, J. M. and Bunn, S. (1999). Dispersal among populations of *Caridina* sp. (decapoda: Atyidae) in coastal lowland streams, South-eastern Queensland, Australia. *Marine and Freshwater Research*, **50:** 681-688.

Wright, S. (1951). The genetical structure of populations. *Annals of Eugenics*, **15:** 1-17.

Wright, S. (1965). The interpretation of population structure by f-statistics with special regard to systems of mating. *Evolution,* **19:** 395-420.

Wright, S. (1978). Evolution and the Genetics of Populations. Vol. 4. Variability within and among Natural Populations. University of Chicago Press, Chicago.

Yang, S. Y., Soule, M. and Gorman, G. C. (1974). *Anolis* lizards of the eastern Caribbean: a case study in evolution. I. Genetic relationships, phylogeny and colonisation sequences of the roquet group. *Systematic Zoology,* **23:** 387-399.

Yoder, A., Irwin, J. A. and Payseur, B. A. (2001). Failure of the ILD to determine data combinability for slow *Loris* phylogeny. *Systematic Biology,* **53:** 408- 424.

Zar, J. H. (1968). Calculation and miscalculation of the allometric equation as a model in biological data. *Bioscience,* **18:** 1118-1120.

Zwanenburg, K.C. T., Benzten, P. and Wright, S. (1992). Mitochondrial DNA differentiation in Western North Atlantic populations of haddock (*Melanogrammus aeglefinus*). *Canadian Journal of Fisheries and Aquatic Sciences*, **49:** 2527- 2537.

Appendix 1. Allele frequencies of the polymorphic loci for the 11 populations in the *P. brincki* complexs. N denotes the sample size.

	Population								
Locus	1	2	3	4	5	6	7	8	9
ARK-1									
(N)	47	10	8	27	30	13	23	5	22
A	.000	.000	.000	.000	1.000	1.000	1.000	1.000	1.000
B	1.000	1.000	1.000	1.000	.000	.000	.000	.000	.000
GPI-1									
(N)	47	10	8	27	30	13	23	5	23
A	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	.968	1.000	1.000	1.000	.983	1.000	.913	.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000
D	.032	.000	.000	.000	.017	.000	.087	.000	.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.000	.000
G	.000	.000	.000	.000	.000	.000	.000	.000	.000
H	.000	.000	.000	.000	.000	.000	.000	.000	.000
I	.000	.000	.000	.000	.000	.000	.000	1.000	1.000
J	.000	.000	.000	.000	.000	.000	.000	.000	.000
ME-1									
(N)	46	4	5	23	18	12	20	5	19
A	.000	.750	.000	.000	.000	.000	.000	1.000	1.000
B	1.000	.250	1.000	1.000	1.000	1.000	1.000	.000	.000
MDH-2									
(N)	45	5	8	27	25	12	23	5	23
A	.978	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	.022	.000	.000	.000	.000	.000	.000	.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000
LDH-2									
(N)	43	9	8	27	29	11	23	5	18
A	1.000	.556	1.000	1.000	.931	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000
D	.000	.444	.000	.000	.069	.000	.000	.000	.000
PGM-1									
(N)	47	9	8	27	27	11	23	5	19
A	.000	.278	.250	.093	.000	.000	.000	1.000	1.000
B	1.000	.722	.750	.907	.926	1.000	1.000	.000	.000
C	.000	.000	.000	.000	.074	.000	.000	.000	.000
GL-1									
(N)	42	10	2	27	28	13	23	5	23
A	.024	.850	1.000	.889	.946	.808	1.000	1.000	.826
B	.976	.000	.000	.000	.000	.000	.000	.000	.022
C	.000	.000	.000	.000	.000	.000	.000	.000	.065
D	.000	.150	.000	.111	.054	.192	.000	.000	.087

Appendix 1. continues.

	Population	
Locus	10	11
ARK-1		
(N)	11	38
A	1.000	.000
B	.000	1.000
GPI-1		
(N)	11	37
A	.000	.000
B	.000	.000
C	.000	.000
D	.000	.000
E	.000	.000
F	.000	.000
G	.000	.000
H	.000	.000
I	1.000	.905
J	.000	.095
ME-1		
(N)	11	31
A	1.000	1.000
B	.000	.000
MDH-2		
(N)	11	28
A	1.000	.946
B	.000	.018
C	.000	.036
LDH-2		
(N)	11	35
A	1.000	1.000
B	.000	.000
C	.000	.000
D	.000	.000
PGM-1		
(N)	11	30
A	1.000	1.000
B	.000	.000
C	.000	.000
GL-1		
(N)	10	36
A	1.000	1.000
B	.000	.000
C	.000	.000
D	.000	.000

Key to populations

1. Blinkwater, 2. Orange kloof, 3. Kirstenbosch, 4. Silvermine, 5. Jonkershoek,

6. Louwrens Rivier, 7. Sir Lowry's Pass, 8. Steenbras, 9. Rooiels, 10. Palmiet and 11.

Fernkloof.

Appendix 2. Allele frequencies for the polymorphic loci among the 28 freshwater crab populations sampled. N denotes the sample size.

	Population								
Locus	1	2	3	4	5	6	7	8	9
GPI-1									
(N)	20	7	15	16	20	12	12	17	16
A	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	.975	1.000	.967	.531	.950	1.000	1.000	1.000	1.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000
D	.000	.000	.033	.000	.000	.000	.000	.000	.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.000	.000
G	.000	.000	.000	.000	.000	.000	.000	.000	.000
H	.000	.000	.000	.000	.000	.000	.000	.000	.000
I	.000	.000	.000	.000	.000	.000	.000	.000	.000
J	.000	.000	.000	.000	.000	.000	.000	.000	.000
K	.025	.000	.000	.469	.050	.000	.000	.000	.000
LT-1									
(N)	20	7	15	16	20	12	12	17	16
A	1.000	1.000	.967	1.000	1.000	1.000	1.000	1.000	.875
B	.000	.000	.033	.000	.000	.000	.000	.000	.125
LT-2									
(N)	20	7	15	16	20	12	12	17	16
A	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	1.000	1.000	.967	1.000	1.000	1.000	1.000	1.000	1.000
C	.000	.000	.033	.000	.000	.000	.000	.000	.000
PGD-1									
(N)	20	7	15	16	20	12	12	17	16
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000
MDH-2									
(N)	11	7	9	10	20	12	12	17	16
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000
IDH-1									
(N)	12	3	12	14	20	12	12	17	16
A	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	1.000	1.000	.958	1.000	1.000	1.000	1.000	1.000	1.000
C	.000	.000	.042	.000	.000	.000	.000	.000	.000
PGM-1									
(N)	19	7	14	16	10	11	5	16	15
A	.079	.429	.786	.313	.000	.227	.000	.000	.000
B	.316	.000	.036	.000	.150	.591	.100	.313	.300
C	.000	.000	.000	.000	.450	.000	.000	.000	.000
D	.605	.571	.179	.688	.250	.182	.900	.594	.600
E	.000	.000	.000	.000	.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.050	.000	.000	.000	.000
G	.000	.000	.000	.000	.000	.000	.000	.000	.000
H	.000	.000	.000	.000	.000	.000	.000	.000	.000
I	.000	.000	.000	.000	.000	.000	.000	.000	.000
J	.000	.000	.000	.000	.000	.000	.000	.000	.000
K	.000	.000	.000	.000	.100	.000	.000	.094	.100
PGM-2									
(N)	17	6	15	16	20	12	12	17	16
A	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 2. continues.

	Populations								
Locus	10	11	12	13	14	15	16	17	18
GL-1									
(N)	20	7	15	16	20	12	12	17	16
A	.950	1.000	.967	1.000	1.000	1.000	1.000	1.000	1.000
B	.000	.000	.033	.000	.000	.000	.000	.000	.000
C	.050	.000	.000	.000	.000	.000	.000	.000	.000
GPI-1									
(N)	25	13	8	12	14	13	14	19	16
A	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	.980	.885	1.000	.958	.964	1.000	1.000	.868	.969
C	.000	.000	.000	.000	.000	.000	.000	.000	.000
D	.020	.115	.000	.000	.000	.000	.000	.132	.031
E	.000	.000	.000	.000	.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.000	.000
G	.000	.000	.000	.000	.000	.000	.000	.000	.000
H	.000	.000	.000	.000	.000	.000	.000	.000	.000
I	.000	.000	.000	.000	.000	.000	.000	.000	.000
J	.000	.000	.000	.000	.000	.000	.000	.000	.000
K	.000	.000	.000	.042	.036	.000	.000	.000	.000
LT-1									
(N)	25	13	8	12	14	13	14	19	16
A	1.000	1.000	1.000	1.000	.857	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.143	.000	.000	.000	.000
LT-2									
(N)	25	13	8	12	14	13	14	19	16
A	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000
PGD-1									
(N)	25	13	8	12	14	13	14	19	16
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.974	1.000
B	.000	.000	.000	.000	.000	.000	.000	.026	.000
MDH-2									
(N)	25	13	8	12	14	13	14	19	16
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000
IDH-1									
(N)	25	13	8	12	14	13	14	19	16
A	.000	.000	.000	.000	.000	.000	.000	.000	.000
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000
PGM-1									
(N)	13	11	8	12	11	9	9	19	16
A	.038	.136	.000	.000	.000	.000	.111	.132	.000
B	.692	.409	.000	.000	.091	.444	.889	.132	.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000
D	.269	.318	1.000	1.000	.909	.556	.000	.737	1.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.000	.000
G	.000	.000	.000	.000	.000	.000	.000	.000	.000
H	.000	.000	.000	.000	.000	.000	.000	.000	.000
I	.000	.000	.000	.000	.000	.000	.000	.000	.000
J	.000	.000	.000	.000	.000	.000	.000	.000	.000
K	.000	.136	.000	.000	.000	.000	.000	.000	.000
GL-1									
(N)	25	13	8	12	14	13	14	19	16
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000

Appendix 2. continues.

	Population							
Locus	19	20	21	22	23	24	25	26
GPI-1								
(N)	20	30	32	13	20	16	12	12
A	.000	.000	.000	.000	.000	.000	.000	.000
B	.925	1.000	1.000	1.000	.950	1.000	1.000	1.000
C	.000	.000	.000	.000	.000	.000	.000	.000
D	.000	.000	.000	.000	.050	.000	.000	.000
E	.000	.000	.000	.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.000
G	.000	.000	.000	.000	.000	.000	.000	.000
H	.000	.000	.000	.000	.000	.000	.000	.000
I	.000	.000	.000	.000	.000	.000	.000	.000
J	.000	.000	.000	.000	.000	.000	.000	.000
K	.075	.000	.000	.000	.000	.000	.000	.000
LT-1								
(N)	20	30	32	13	20	16	12	12
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000
LT-2								
(N)	20	16	16	13	20	16	12	12
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000
PGD-1								
(N)	20	30	32	13	20	16	12	12
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000
MDH-2								
(N)	20	30	32	13	20	16	12	12
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000
IDH-1								
(N)	20	30	32	13	20	16	12	12
A	.000	.000	.000	.000	.000	.000	.000	.000
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
C	.000	.000	.000	.000	.000	.000	.000	.000
IDH-2								
(N)	20	30	32	13	20	16	12	12
A	.000	.000	.000	.000	.000	.000	.000	.000
B	.000	.000	.000	.000	.000	.000	.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000
D	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
MPI-1								
(N)	20	30	32	13	20	16	12	12
A	.000	.000	.000	.000	.000	.000	.000	.000
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
PGM-1								
(N)	9	13	16	8	15	16	12	12
A	.000	.000	.000	.000	.000	.031	.000	.000
B	.111	.000	.938	.563	.000	.313	.000	.083
C	.000	.077	.000	.000	.000	.000	.000	.000
D	.889	.923	.063	.438	1.000	.656	1.000	.917
E	.000	.000	.000	.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.000
G	.000	.000	.000	.000	.000	.000	.000	.000
H	.000	.000	.000	.000	.000	.000	.000	.000
I	.000	.000	.000	.000	.000	.000	.000	.000
J	.000	.000	.000	.000	.000	.000	.000	.000
K	.000	.000	.000	.000	.000	.000	.000	.000
GL-1								
(N)	20	30	32	13	20	16	12	12
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000	.000	.000	.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000

Appendix 2. continues.

	Population	
Locus	27	28
GPI-1		
(N)	16	15
A	.000	.000
B	.938	1.000
C	.000	.000
D	.000	.000
E	.000	.000
F	.000	.000
G	.000	.000
H	.000	.000
I	.000	.000
J	.000	.000
K	.063	.000
LT-1		
(N)	16	15
A	1.000	1.000
B	.000	.000
LT-2		
(N)	16	15
A	1.000	1.000
B	.000	.000
C	.000	.000
PGD-1		
(N)	16	15
A	1.000	1.000
B	.000	.000
MDH-2		
(N)	16	15
A	.969	.967
B	.031	.033
IDH-1		
(N)	16	15
A	.000	.000
B	1.000	1.000
C	.000	.000
PGM-1		
(N)	7	12
A	.071	.000
B	.143	.708
C	.000	.000
D	.786	.292
E	.000	.000
F	.000	.000
G	.000	.000
H	.000	.000
I	.000	.000
J	.000	.000
K	.000	.000
GL-1		
(N)	16	15
A	1.000	1.000
B	.000	.000
C	.000	.000

Key to populations

1 Vaal B, 2 Shingwedzi, 3 Mbewedi, 4 Mutamba, 5 Edenvale, 6 Hex River, 7 Sand River, 8 Suikerbosrand Nature Reserve, 9 Elands River, 10 Sterkstroom, 11 Haterbeespoortdam, 12 Vredespruit, 13 Frankfurt, 14 Crocodile River, 15 Zout River,

16 Rustenburg Nature Reserve, 17 Pienaars River, 18 Willem Pretorius Gamepark, 19 Upington, 20 Lenasia, 21 Olivantsvlei , 22 Blesbokspruit, 23 Vereniging, 24 Vals River, 25 Renosterspruit, 26 Suikerbosrand River, 27 Klip River and 28 Villiers.

Appendix 3. Allele frequencies for the 11 populations sampled, N denotes the sample size.

	Population								
Locus	1	2	3	4	5	6	7	8	9
ARK-1									
(N)	40	16	49	28	3	4	29	15	44
100	.000	.000	.000	1.000	1.000	1.000	1.000	1.000	.966
75	1.000	1.000	1.000	.000	.000	.000	.000	.000	.034
IDH-2									
(N)	40	16	48	28	3	4	29	15	44
180	.000	.000	.000	.000	.000	.000	.914	1.000	1.000
100	.000	.031	.000	.000	.000	.000	.000	.000	.000
60	1.000	.969	1.000	1.000	1.000	1.000	.000	.000	.000
45	.000	.000	.000	.000	.000	.000	.086	.000	.000
LT-1									
(N)	31	16	48	27	3	3	29	15	44
130	1.000	.938	1.000	1.000	1.000	.667	.862	.900	.955
105	.000	.000	.000	.000	.000	.000	.000	.000	.045
100	.000	.000	.000	.000	.000	.000	.052	.033	.000
85	.000	.063	.000	.000	.000	.333	.069	.067	.000
60	.000	.000	.000	.000	.000	.000	.017	.000	.000
GPI-1									
(N)	40	15	49	28	3	4	29	15	44
180	1.000	.967	.990	.839	1.000	1.000	.983	1.000	.989
170	.000	.000	.000	.036	.000	.000	.017	.000	.000
155	.000	.000	.000	.125	.000	.000	.000	.000	.000
100	.000	.000	.000	.000	.000	.000	.000	.000	.011
-50	.000	.033	.010	.000	.000	.000	.000	.000	.000
ME-1									
(N)	35	16	48	28	3	4	29	15	44
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.955
90	.000	.000	.000	.000	.000	.000	.000	.000	.045
HEX-1									
(N)	39	16	48	26	3	4	12	15	44
105	1.000	1.000	1.000	.962	.333	1.000	.917	1.000	.977
100	.000	.000	.000	.038	.000	.000	.083	.000	.023
90	.000	.000	.000	.000	.167	.000	.000	.000	.000
85	.000	.000	.000	.000	.500	.000	.000	.000	.000
MDH-1									
(N)	38	16	49	28	3	4	29	15	20
105	1.000	1.000	1.000	1.000	1.000	1.000	.000	.000	.000
100	.000	.000	.000	.000	.000	.000	1.000	1.000	1.000
MDH-2									
(N)	40	16	49	28	3	4	29	15	44
105	.000	.000	.000	.000	.000	.000	.966	1.000	1.000
100	1.000	1.000	1.000	1.000	1.000	1.000	.034	.000	.000
IDH-1									
(N)	19	16	43	19	3	3	25	15	43
150	.000	.000	.012	.421	.000	.833	.420	.200	.163
135	.000	.000	.000	.000	.000	.000	.000	.000	.000
130	.921	.813	.988	.579	1.000	.167	.560	.733	.837
120	.026	.000	.000	.000	.000	.000	.000	.000	.000
100	.053	.188	.000	.000	.000	.000	.000	.067	.000

Appendix 3. continues.

	Population								
Locus	1	2	3	4	5	6	7	8	9
LDH-1									
(N)	32	16	48	28	3	4	29	15	44
115	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.933	1.000
110	.000	.000	.000	.000	.000	.000	.000	.033	.000
100	.000	.000	.000	.000	.000	.000	.000	.033	.000
PGM-1									
(N)	37	16	48	28	3	4	29	15	44
110	.000	.000	.010	.000	.000	.000	.000	.000	.000
100	1.000	1.000	.990	1.000	1.000	.000	.000	.000	.000
90	.000	.000	.000	.000	.000	.000	1.000	1.000	1.000
80	.000	.000	.000	.000	.000	1.000	.000	.000	.000
GL-1									
(N)	38	16	47	26	3	4	17	14	44
110	.368	.938	.351	.058	.500	.875	.000	.607	.920
100	.000	.000	.000	.000	.000	.000	1.000	.000	.000
95	.632	.063	.649	.942	.500	.125	.000	.393	.080
6-PGD									
(N)	28	16	48	25	3	4	29	15	44
105	.000	.000	.000	.000	.000	.000	.000	.000	.057
100	.000	.000	.000	.000	.000	.000	.983	1.000	.943
95	1.000	1.000	1.000	1.000	1.000	1.000	.017	.000	.000
PHP-1									
(N)	24	16	49	8	3	4	29	15	44
105	.000	.000	.000	.000	.000	.000	1.000	1.000	.977
100	1.000	.938	1.000	.000	.000	1.000	.000	.000	.000
95	.000	.000	.000	.000	.000	.000	.000	.000	.011
90	.000	.063	.000	1.000	1.000	.000	.000	.000	.011

Appendixs 3. continues.

	Population	
Locus	10	11
ARK-1		
(N)	15	32
100	1.000	1.000
75	.000	.000
IDH-2		
(N)	15	32
180	1.000	1.000
100	.000	.000
60	.000	.000
45	.000	.000
LT-1		
(N)	15	32
130	1.000	1.000
105	.000	.000
100	.000	.000
85	.000	.000
60	.000	.000
GPI-1		
(N)	15	32
180	1.000	.625
170	.000	.375
155	.000	.000
100	.000	.000
-50	.000	.000
ME-1		
(N)	15	32
100	1.000	1.000
90	.000	.000
HEX-1		
(N)	15	32
105	.867	1.000
100	.033	.000
90	.100	.000
85	.000	.000
MDH-1		
(N)	15	32
105	1.000	.906
100	.000	.094
MDH-2		
(N)	15	32
100	.933	1.000
105	.067	.000
IDH-1		
(N)	15	32
150	.967	.969
135	.000	.000
130	.033	.031
120	.000	.000
100	.000	.000

Appendixs 3. continues.

	Population	
Locus	10	11
LDH-1		
(N)	15	32
115	1.000	1.000
110	.000	.000
100	.000	.000
PGM-1		
(N)	15	31
110	1.000	.871
100	.000	.048
90	.000	.081
80	.000	.000
GL-1		
(N)	15	32
110	.833	.875
100	.000	.000
95	.167	.125
6-PGD		
(N)	15	15
105	1.000	.167
100	.000	.833
95	.000	.000
PHP-1		
(N)	15	17
105	1.000	1.000
100	.000	.000
95	.000	.000
90	.000	.000

Key to populations

1 Oliviershoek, 2 Gudu falls, 3 Mahai, 4 Cathedral Peak A, 5 Doreen Falls A, 6 Cathedral Peak B, 7 Doreen Falls B, 8 Monks Cowl, 9 Injasuti, 10 Loteni and 11 Kamberg.

Appendix 4. Morphological characters for southern African *Potamonautes* phylogeny

1. Each character is numbered and described, followed by the character state where 0 = presumed plesiomorphy while 1 and 2 are the presumed apomorphie.

1. Antero-lateral margin of the carapace (where post frontal crest meets the epibranchial corner) with a distinct epibranchial tooth: large triangle shapes tooth present (0); tooth reduced, spine like (1); tooth absent (2)
2. Teeth on the antero-lateral margin of the carapace posterior to epibranchial tooth: a large number of well defined teeth present (0); granulated or smooth (1)
3. Postfrontal crest slopes posteriorly to form a distinct sinus present at the junction between the postfrontal crest and the epibranchial tooth: present (0); absent (1)
4. Exorbital tooth: sharp and well defined (0); smooth (1)
5. Postfrontal crest: well defined, deep and complete (0); eroded (1)
6. Postfrontal crest: with a distinct postorbital notch present (0); absent (1)
7. Exorbital sinus: present (0); absent (1)
8. First carpal tooth on the carpus of the cheliped: long, sharp and pointed (0); carpal tooth reduced (1)
9. Second carpal tooth: well-defined (0); reduced (1)
10. Lateral inferior margin of the merus of the cheliped: lined with small tooth (0); tooth absent (1)
11. Major cheliped: flat and shovel shaped / spatulated (0); broad and elongated (1)
12. Dactylus of the major cheliped: broad and flat (0); long slender (1)
13. Gape of cheliped when closed: no space between dactylus and propodus (0); with a wide space (1)

(CWA/CL) three data partitions could be identified. Species possessing large carapace widths relative to the carapace length, represents the plesiomorphic state (20.0), while species with medium and small carapace widths represents the apomorphic state (20.1). Cumberlidge (1999a) suggests that the species with large CWA relative to CL would be more primitive, while the species with the inverse condition would be the apomorphic state.

Similarly the carapace widest width (CWW) was divided by CL, and the two distinct groups could be detected, with large bodied species representing the pleisiomorphic condition (21.1), while the small bodied forms the apomorphic state (21.1). When the width of the carapace posteriorly (CWP) was divided by its length two distinct morphotypes could be discerned, large bodies species were considered to be plesiomorphic (22.0) while small bodied species were apomorphic (22.1).

Carpal teeth and the lateral inferior margin (characters 8, 9 and 10)

Dentition patterns always provide good reference points for the discerning freshwater crab species (Cumberlidge, 1999a). The carpal teeth in the *Potamonautes* are diagnostic for discerning species. In *P. calcaratus*, the first and second carpal tooth are long, sharp and pointed (the plesiomorphic state, 8.0) while, in the remainder of the species the carpal teeth are reduced (representing the apomorphic state, 8.1). In *P. calcaratus* the second carpal tooth is well developed representing the plesiomorphic state (9.0), while in other species the tooth is reduced (9.1). Again, in *P. calcaratus*, the inferior margin of the merus of the cheliped is lined with small teeth (the plesiomorphic state, 10.0) the absent of teeth is the apomorphic state (10.1).

14. Terminal segment of the mandibular palp: a ridge or flange absent (0); a ridge or flange present (1)
15. Terminal segment of pleopod 1: curved sharply outward towards the vertical (0) straight or slightly curved (1)
16. Carpus of pereopods 2-5 with a distinct spine: present (0); absent (1)
17. Merus of third maxilliped with a distinct groove for the maxilliped: present (0); absent (1)
18. Carapace length, < 35mm (0); >2 5 mm but < 35 mm (1); < 25 mm (2)
19. Carapace height ratio(ch/cl) < 0.45 (0); > 0.45 < 0.45 (1); > 0.55
20. Carapace widest width anterior ratio (cwa/cl), < 0.90 (0); > 0.90 < 0.95 (1); > 0.95
21. Carapace widest width ratio (cww/cl), < 1.35 (0); > 1.35 (1)
22. Carapace widest width posterior ratio (cwp/cl), < 0.95 (0); > 0.95 (1)
23. Ratio of periopod 5 merus width to periopod 5 merus length (p5mw/p5ml), < 0.35 (0); > 0.35 (1)
24. Ratio of periopod 5 propodus width to peripod 5 propodus length (p5pw/p5pl), < 0.50 (0); > 0.50 (1)
25. Ratio of periopod 2 merus width to periopod 2 merus length ratio (p2mw/p2ml), < 0.35 (0); > 0.35 (1)
26. Ratio of periopod 2 propodus width to periopod 2 propodus length (p2pw/p2pl), < 0.50 (0); > 0.50 (1)

Appendix 5. Morpholgical character selection and coding.

In the following section, the morphological characters used in this study are described. Character states are indicated in the text by numbers in parentheses, presumed plesiomorphic state are coded 0, while presumed apomorphic characters have values > 0. The numbered states do not imply a transformation series.

Carapace characteristics (characters 1-7; 18-22)

Diagnostic morphological characters derived from the carapace structure have been used extensively in the classification of the *Potamonautes*. The characters and the states are derived from those proposed by Bott (1955) and Cumberlidge (1999a). The ornamentation of the anterolateral margin of the carapace has been particularly useful in discriminating freshwater crab species. Species with well-defined dentition on the anterolateral margin of the carapace (e.g. *P. warreni, P. unispinus, P. bayonianus, P. parvispina* and *P. calcaratus*) are thought to represent the plesiomorphic state (1.0) (Cumberlidge, 1999a). Species with a granular (e.g. *P. perlatus, P. sidneyi, P. granularis* and *P.lividus*) to smooth (e.g. *P. brincki, P. parvicorpus, P. depressus* and *P. clarus*) anterolateral margin are thought to represent the apomorphic state (1.1; 1.2). For example, Bott (1955) and Cumberlidge (1999a) remarked that species with large well-defined teeth are almost exclusively associated with river systems. These teeth are thought to have served as protection against soft-mouthed predators, and would hinder the movement of these animals on land. In contrast, species with a granulated or smooth are able of living in the terrestrial environment, and nearly always lack spines. The transition from a fully aquatic lifestyle to a terrestrial habitat is thought to have led to this shift in the body morphology (Bott, 1955; Cumberlidge,

1999a).

Where a number of teeth occur on the anterolateral margins, posterior to the epibranchial tooth, this is though to be the pleisomorphic (2.0) state (e.g. *P. warreni,* and *P. dentatus*), while the absence of a tooth (e.g. *P. perlatus, P. sidneyi, P. granularis, P.lividus, P. brincki, P. parvicorpus, P. depressus* and *P. clarus*) is thought to be the apomorphic state (2.1). This is also seen as an adaptation from a riverine existence to a terrestrial mode of life (Cumberlidge, 1999a). Where the postfrontal crest slopes posteriorly to form a distinct sinus at the junction between the crest and the epibranchial tooth is thought to represents the plesiomorphic state (3.0) (e.g. *P. dentatus* and *P. calcaratus*), while the absence of this character is though to be apomorphic (3.1). The presence of these sinuses at the junction of the postfrontal crest is thought by Bott (1955) and Cumberlidge (1999a) to be the ancestral state and this character would serve no value to facilitate the transition from a fully aquatic mode to a terrestrial lifestyle. When the exorbital tooth is sharp and well defined this is the plesiomorphic state (4.0) (in all large bodied riverine crabs and in *P. lividus*), a smooth exorbital tooth the apomorphic state (4.1). Both Bott (1955) and Cumberlidge (1999a) are of the opinion that these changes reflect on the adaptation of species to a terrestrial lifestyle. Sharp exorbital teeth would serve as defence against aquatic predators is the plesiomorphic state, the absence of teeth as evident in most terrestrial species would be the apomorphic state (Cumberlidge, 1999a).

Among most large bodied species, the postfrontal crest is well defined, this is the plesiomorphic state (5.0), while an eroded postfrontal crest carapace more typical of small bodied stream and terrestrial species represents the apomorphic state (5.1).

Cumberlidge (1999a) sees these adaptations as a direct result of the in the increase of the gill chambers that is required when animals invade the terrestrial environment, this character is related to the chest depth (CH- character 19). The carapace depth (defined by the ratio, carapace height over carapace length (CH/CL), could be divided into two major partitions, where the species with medium to small carapace heights represents the plesiomorphic state (19.0), while species with large faulted carapaces > 0.45 represents the apomorphic condition (19.1). Cumberlidge (1999a) noted the same pattern and character states among West African freshwater crab species. He clearly sates that the high, arched carapace is related to increase air breathing, while the decrease in carapace height is the result of a fully aquatic lifestyle. Where the postfrontal crest possesses a distinct postorbital notch this is the plesiomorphic state (6.0), while the absence of a notch represents the advanced state (6.1).

Bott (1955) noted that the presence of a exorbital sinus is a well defined character, here, the presence of this character is thought to represent a pleiseiomorphic state (7.0) while its absence reflects the apomorphic state (7.1). Based on the morphometric data, at least three distinct size classes could be determined where large bodied species (with carapace length – CL >35mm) represents the plesiomorphic state (18.0), and medium (> 25mm but < 35mm) to small bodied species (<25mm) represents the apomorphic state (18.1). The large bodied species associated with river systems, are thought to be the plesiomorphic state (Bott, 1955; Cumberlidge, 1999a), while small bodied species are thought to represent the apomorphic condition.

When the carapace widest width anterior was divided (CWA) by carapace length CL

Cheliped characteristics (characters11, 12 and 13)

Cheliped structure has been used extensively in the description of freshwater crabs (Cumberlidge, 1999a). In all riverine species, the chelipeds are "swollen" while the dactylus and the propodus are long and arched, representing the apomorphic state (11.1; 12.1). In *P. calcaratus* the chelipeds are shovel shaped and dactylus and the propodus are broad and flat representing the pleisomorphic condition (11.0; 12.0). When the gape of the chelipeds is closed (dactylus against propodus) and no space is evident between the "fingers" of the cheliped this is the plesiomorphic state (13.0), while a highly arched cheliped with a open gape is the apomorphic state. Cumberlidge (1999a) is of the opinion that the plesiomorphic states are retained from a burrowing marine ancestral species.

Terminal segment of the mandibular palp (character 14)

The absence of a ridge of the terminal segment of the mandibular palp is the plesiomorphic state (14.0), while the presence of the ridge or flange is the autapomorphic state as present in *P. brincki*. This autapomorphy was excluded from the analysis.

Pleopod 1 or gonopod 1(character 15)

The structure of pleopod 1 is among the most diagnostic of all the characteristics, to discern freshwater crab species (Cumberlidge, 1999a). However, in the southern African species, the structure of pleopod 1 appears relatively conserved. The terminal segment of pleopod 1 falls into two discreet groups and it is either curved sharply (the

plesiomorphic state as in *P. calcaratus* 15.0) or it is straight and slightly curved (the apomorphic state, 15. 1).

Pereopods (16; 23 – 26)

In *P. calcaratus* the carpus of the walking limbs does not possess spines, this is the plesiomorphic state (16.0), while species that lack these spines represent the apomorphic state (16.1). The presence of spines on the walking limbs is clearly a plesiomorphic character according to Cumberlidge (1999). The pereopods shape has also proven to useful in discriminating species pairs (e.g. Daniels *et al.* 1998). Species with long, narrow limbs represent the plesiomorphic state (23. 0; 24.0; 25; 0; 26.0) while species with broad large limbs are thought to represent the apomorphic state (23.1; 24.1; 25.1, 26.1). Cumberlidge (1999a) based these characters state changes on the transition of the ancesteral species from a fully aquatic animal to a animal adapted to terrestrial living.

Appendix 6. Allele frequencies of the polymorphic loci for the 13 freshwater crabs species. N denotes the sample size.

	Species								
Locus	1	2	3	4	5	6	7	8	9
ARK-1									
(N)	172	76	99	105	48	63	92	69	39
A	.384	.500	1.000	.000	1.000	1.000	1.000	1.000	1.000
B	.616	.500	.000	1.000	.000	.000	.000	.000	.000
IDH-2									
(N)	160	55	99	104	48	63	90	66	39
A	.000	.000	1.000	.000	1.000	.000	.000	.000	.000
B	.000	.000	.000	.000	.000	1.000	.511	.000	1.000
C	1.000	1.000	.000	.000	.000	.000	.000	1.000	.000
D	.000	.000	.000	.005	.000	.000	.183	.000	.000
E	.000	.000	.000	.995	.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.000	.000	.306	.000	.000
GPI-1									
(N)	172	76	99	104	48	63	92	69	39
A	.000	.000	.000	.990	1.000	.000	.000	.000	.000
B	.965	.000	.000	.000	.000	.056	.967	.007	1.000
C	.000	.000	.000	.000	.000	.143	.011	.000	.000
D	.035	.000	.000	.000	.000	.000	.022	.971	.000
E	.000	.000	1.000	.010	.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.022	.000
G	.000	.000	.000	.000	.000	.000	.000	.000	.000
H	.000	.000	.000	.000	.000	.698	.000	.000	.000
I	.000	.954	.000	.000	.000	.000	.000	.000	.000
J	.000	.046	.000	.000	.000	.000	.000	.000	.000
K	.000	.000	.000	.000	.000	.103	.000	.000	.000
ME-1									
(N)	142	66	99	99	48	63	92	69	39
A	.021	1.000	.000	1.000	.750	1.000	.000	1.000	1.000
B	.979	.000	1.000	.000	.250	.000	1.000	.000	.000
MDH-1									
(N)	158	71	99	103	48	63	92	69	39
A	1.000	1.000	1.000	1.000	.958	.992	.995	1.000	1.000
B	.000	.000	.000	.000	.000	.008	.005	.000	.000
C	.000	.000	.000	.000	.010	.000	.000	.000	.000
D	.000	.000	.000	.000	.031	.000	.000	.000	.000
MDH-2									
(N)	159	67	99	105	48	63	92	69	39
A	.994	.978	1.000	.000	.938	1.000	1.000	.949	.987
B	.006	.007	.000	.000	.000	.000	.000	.051	.013
C	.000	.015	.000	1.000	.063	.000	.000	.000	.000
D	.000	.000	.000	.000	.000	.000	.000	.000	.000
IDH-1									
(N)	144	64	99	78	48	44	92	29	34
A	.000	.000	.000	.006	.979	.000	.000	.000	.000
B	1.000	1.000	.000	.000	.000	1.000	.000	.966	1.000
C	.000	.000	.000	.936	.021	.000	.000	.034	.000
D	.000	.000	1.000	.006	.000	.000	1.000	.000	.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.000
F	.000	.000	.000	.051	.000	.000	.000	.000	.000
LT-2									
(N)	155	57	99	93	48	63	92	63	39
A	.000	.000	.000	.000	.969	.000	.859	.000	.000
B	.000	.000	.000	.000	.000	.000	.109	.000	.000
C	.000	.000	.000	.989	.031	1.000	.000	.000	1.000
D	1.000	1.000	.000	.000	.000	.000	.000	.984	.000
E	.000	.000	.000	.011	.000	.000	.033	.016	.000
F	.000	.000	.000	.000	.000	.000	.000	.000	.000
G	.000	.000	1.000	.000	.000	.000	.000	.000	.000

Appendix 6. continues.

LDH-2									
(N)	164	69	99	96	48	63	92	64	39
A	.963	1.000	.000	1.000	1.000	1.000	1.000	1.000	1.000
B	.000	.000	1.000	.000	.000	.000	.000	.000	.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000
D	.037	.000	.000	.000	.000	.000	.000	.000	.000
PGM-1									
(N)	166	65	98	101	48	38	92	60	39
A	.042	1.000	.000	.005	1.000	.276	.076	.000	.026
B	.946	.000	.000	.995	.000	.658	.761	1.000	.974
C	.012	.000	.240	.000	.000	.066	.163	.000	.000
D	.000	.000	.760	.000	.000	.000	.000	.000	.000
GL-1									
(N)	159	73	99	101	47	63	92	64	39
A	.601	.945	.000	.450	.915	.000	.000	.016	.000
B	.346	.007	.000	.000	.032	1.000	.989	.000	1.000
C	.000	.021	.000	.000	.053	.000	.000	.961	.000
D	.053	.027	.000	.000	.000	.000	.000	.023	.000
E	.000	.000	.000	.000	.000	.000	.011	.000	.000
F	.000	.000	.000	.550	.000	.000	.000	.000	.000
G	.000	.000	1.000	.000	.000	.000	.000	.000	.000
MPI-1									
(N)	147	60	99	104	48	63	92	69	39
A	.000	.000	.000	1.000	.865	.000	.000	.000	.000
B	.986	1.000	1.000	.000	.000	1.000	1.000	1.000	1.000
C	.000	.000	.000	.000	.000	.000	.000	.000	.000
D	.014	.000	.000	.000	.000	.000	.000	.000	.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.135	.000	.000	.000	.000
PGM-2									
(N)	155	56	99	101	48	63	92	49	39
A	.987	1.000	.000	1.000	1.000	1.000	1.000	1.000	1.000
B	.013	.000	1.000	.000	.000	.000	.000	.000	.000
LT-1									
(N)	55	52	99	40	48	9	92	49	39
A	1.000	1.000	.000	1.000	1.000	.000	.000	1.000	.000
B	.000	.000	.000	.000	.000	1.000	1.000	.000	1.000
C	.000	.000	1.000	.000	.000	.000	.000	.000	.000
D	.000	.000	.000	.000	.000	.000	.000	.000	.000
E	.000	.000	.000	.000	.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.000	.000	.000	.000	.000

Appendix 6. continues.

	Species			
Locus	10	11	12	13
ARK-1				
(N)	45	39	39	39
A	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000
IDH-2				
(N)	45	39	39	39
A	.000	.000	.000	.000
B	1.000	.000	.000	.000
C	.000	.000	.000	.000
D	.000	1.000	1.000	1.000
E	.000	.000	.000	.000
F	.000	.000	.000	.000
GPI-1				
(N)	45	39	39	39
A	.000	.000	.000	.000
B	.922	1.000	1.000	.000
C	.078	.000	.000	.000
D	.000	.000	.000	1.000
E	.000	.000	.000	.000
F	.000	.000	.000	.000
G	.000	.000	.000	.000
H	.000	.000	.000	.000
I	.000	.000	.000	.000
J	.000	.000	.000	.000
K	.000	.000	.000	.000
ME-1				
(N)	45	39	39	39
A	.000	.000	.000	.000
B	1.000	1.000	1.000	1.000
MDH-1				
(N)	45	39	39	39
A	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000
C	.000	.000	.000	.000
D	.000	.000	.000	.000
MDH-2				
(N)	45	39	39	39
A	.967	1.000	1.000	1.000
B	.000	.000	.000	.000
C	.022	.000	.000	.000
D	.011	.000	.000	.000
IDH-1				
(N)	43	39	39	39
A	.000	.000	.000	.000
B	.000	1.000	1.000	1.000
C	.000	.000	.000	.000
D	.081	.000	.000	.000
E	.919	.000	.000	.000
F	.000	.000	.000	.000
LT-2				
(N)	45	39	39	39
A	.967	.000	1.000	1.000
B	.033	1.000	.000	.000
C	.000	.000	.000	.000
D	.000	.000	.000	.000
E	.000	.000	.000	.000
F	.000	.000	.000	.000
G	.000	.000	.000	.000

Appendix 6. continues.

	Species			
Locus	10	11	12	13
LDH-2				
(N)	45	39	39	39
A	1.000	1.000	1.000	1.000
B	.000	.000	.000	.000
C	.000	.000	.000	.000
D	.000	.000	.000	.000
PGM-1				
(N)	45	39	39	39
A	.100	.051	.051	.000
B	.856	.423	.423	.474
C	.000	.000	.000	.000
D	.044	.526	.526	.526
GL-1				
(N)	45	38	38	38
A	.000	.961	.961	.961
B	1.000	.000	.000	.000
C	.000	.039	.039	.039
D	.000	.000	.000	.000
E	.000	.000	.000	.000
F	.000	.000	.000	.000
G	.000	.000	.000	.000
MPI-1				
(N)	45	39	39	39
A	.000	.000	.000	.000
B	1.000	1.000	1.000	1.000
C	.000	.000	.000	.000
D	.000	.000	.000	.000
E	.000	.000	.000	.000
F	.000	.000	.000	.000
PGM-2				
(N)	45	39	39	39
A	.022	.000	.103	.000
B	.978	1.000	.897	1.000
LT-1				
(N)	45	39	39	39
A	.000	1.000	1.000	.000
B	1.000	.000	.000	.000
C	.000	.000	.000	.000
D	.000	.000	.000	.000
E	.000	.000	.000	.000
F	.000	.000	.000	1.000

Key to species

1. *P. parvicorpus,* 2. *P. brincki,* 3. *P. calcaratus,* 4. *P. clarus,* 5. *P. depressus*

6 *P. granularis*, 7 *P. lividus,* 8. *P. parvispina,* 9. *P. perlatus,* 10. *P. sidneyi*

11. *P. unispinus,* 12 *P. warreni* and 13. *P. dentatus*