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EXAMINING THE GENETIC STRUCTURE AMONG POPULATIONS OF THE COMMON CAPE RIVER CRAB *POTAMONAUTES PERLATUS* FROM RIVER SYSTEMS IN SOUTH AFRICA REVEALS HYDROGRAPHIC BOUNDARIES

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A B S T R A C T

The genetic structure of the common Cape river crab *Potamonautes perlatus* (Decapoda: Potamonautidae) was assessed among 21 populations from major drainage systems in South Africa using allozymes and sequence data from two mitochondrial genes (16S rRNA and COI). Protein gel electrophoresis of 15 loci encoding 11 enzyme systems revealed limited genetic variation in the Berg and Olifants drainages. However, in the Gamtoos and Breë river systems, and over all populations, moderate levels of variation were evident from the F_{ST} values. Gene-flow estimates revealed high levels of dispersal within and between drainages. In contrast, phylogenetic analysis of the 16S rRNA mtDNA sequence data from 10 representative populations revealed two distinct clades. One consisted of all the rivers that drain the western escarpment, and another consisted of rivers that drain the southern escarpment. The COI mtDNA and a combined sequence data analysis consistently placed the western flowing rivers in a distinct clade. The split into two clades coincided with the western and southern drainages with the Cape Fold mountains being the barrier that separates the drainage basins. These results are discussed in light of possible geological factors that could have sculpted the present genetic patterns. Allozyme and sequence data were generally complementary at a small spatial scale (within drainages), where both markers indicated the absence of genetic structure. However, at larger spatial scales, the sequence data proved superior at detecting genetic variation.

Freshwater crabs are widely distributed in South Africa where they can be found in a variety of aquatic habitats ranging from rivers, streams, and lakes to swamp forests. While some species have specific habitat requirements restricting their range, other species are able to tolerate a wide range of habitat types that make them particularly suited to studying levels of gene flow between geographically isolated populations. Freshwater crabs lack larval stages; however, they produce a large number of juveniles (more than 850 eggs per female were recorded in *Potamonautes sidneyi*) (Raubenheimer, 1986). The adults are thought to be amphibious, with a relatively high dispersal capacity, and are frequently seen on foraging excursions on land. Collectively, these factors suggest low levels of genetic variation within river systems and pronounced levels of genetic variation between river systems. However, various patterns of genetic variation exist among freshwater crabs and appear to be decoupled from assumed dispersal capacity and habitat requirements. For example, Daniels *et al*. (2002a) demonstrated, using mt DNA sequence data and allozymes, that for a semi-terrestrial species such as *Potamonautes calcaratus*, populations were genetically moderately structured despite minimal distances between populations. In contrast, an allozyme study by Daniels *et al*. (1998) demonstrated genetic invariance both within and among populations of *P. parvispina* from two geographically isolated drainage systems. These studies indicate that the population genetic structure of each species merits an independent assessment to quantify gene flow among conspecific populations. While allozyme studies have been commonly used in population genetic studies in aquatic invertebrates (Daniels *et al*., 1999a, b; Hughes *et al*., 1995, 1996), mitochondrial DNA sequence data have been less frequently used to examine genetic variation (Bilton *et al*., 2001). The complementary use of molecular markers with different mutation rates is particularly valuable in disentangling historic and contemporary levels of gene flow (Bilton *et al*., 2001).

In the present study, genetic markers are used to explore levels of gene flow among populations of *P. perlatus* in South Africa. *Potamonautes perlatus* has one of the broadest geographic distributions of freshwater crabs in

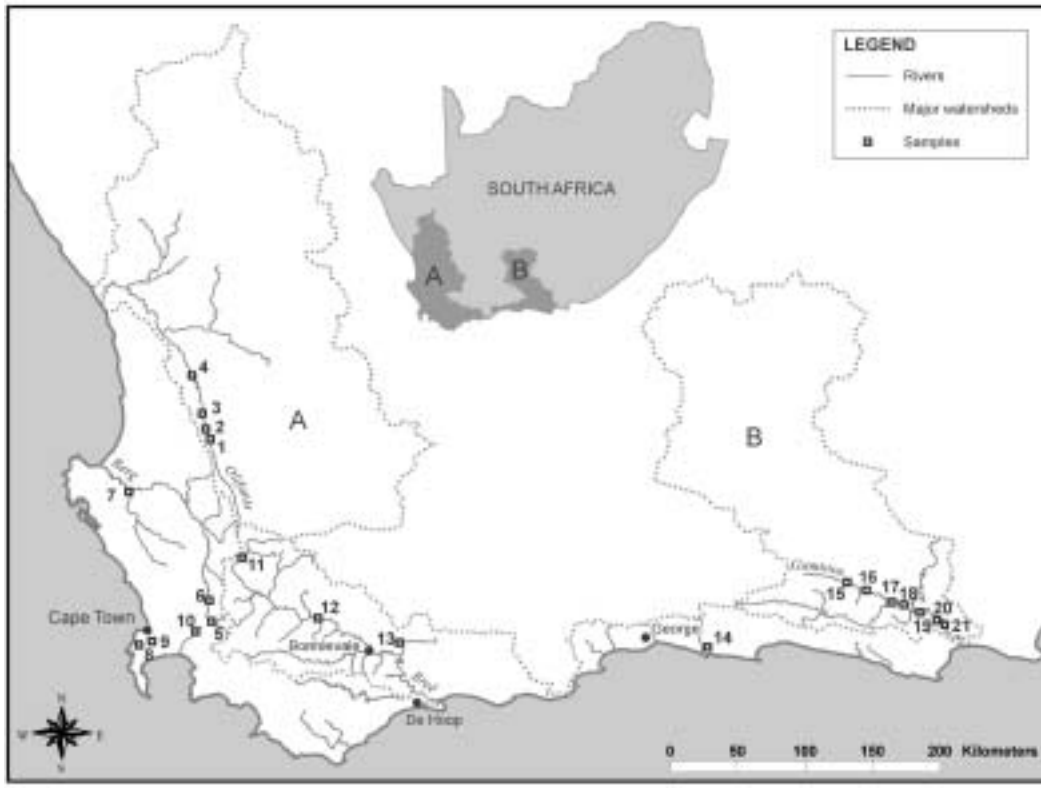

Fig. 1. The localities and major drainages sampled in this study, with *n* denoting the sample sizes. (1) Citrusdal ($n = 10$); (2) Boontjieskloof ($n = 19$); (3) Kriedouwkrans ($n = 23$); (4) Clanwilliam ($n = 20$); (5) Paarl ($n = 19$); (6) Tunnel Terminals ($n = 23$); (7) Water Station ($n = 20$); (8) Liesbeek ($n = 10$); (9) Tokai ($n = 30$); (10) Stellenbosch ($n = 25$); (11) Bainskloof ($n = 15$); (12) Robertson ($n = 10$); (13) Tradouws Pass ($n = 30$); (14) Tsitsikamma ($n = 10$); (15) Sandriver ($n = 10$); (16) Bosdorp ($n = 28$); (17) Smithskraal ($n = 16$); (18) Kleinplaats ($n = 20$); (19) Poortjies ($n = 21$); (20) Patensie ($n = 18$); and (21) Hankey ($n = 25$). The De Hoop and Bonnievale populations, for which only sequence data were collected, are also noted on the map.

the region (Barnard, 1950). This species occurs from Clanwilliam in the Western Cape, and its distribution extends northwards and eastwards into the Southern Cape, where it has been reported to occur at Belmont (Gouws *et al*., 2002); the range stretches nearly 900 km. Stewart (personal communication) reported that a morphological variety of *P. perlatus* which appears distinctly yellow in color and may represent a distinct species occurs in the Gamtoos River in the Southern Cape. Previous genetic studies on *P. perlatus* have focused exclusively on the genetic structure in the Berg and Olifants drainage systems (Daniels *et al*., 1999a, b) and to date have not explored levels of gene flow across large geographic distances. The present study has two objectives: firstly, the degree of population structure both within drainages and over the distribution range of the species is examined with the use of allozyme electrophoresis and sequence data; secondly, the systematic status of the yellow populations of *P. perlatus* collected from the Gamtoos River in the Southern Cape is examined.

Materials and Methods

Sample Collection

Potamonautes perlatus were collected from 21 localities throughout its distribution range in the Western and Southern Cape Province, South Africa (Fig. 1). These included populations from four major drainages: the Berg River (Paarl, Tunnel Terminals, and Water Station); the Breë River (Bainskloof, Robertson, and Tradouws Pass); the Olifants River (Citrusdal, Boontjieskloof, Kriedouwkrans, and Clanwilliam); and the Gamtoos River—the yellow colored crabs (Sandriver, Bosdorp, Smithskraal, Kleinplaats, Poortjies, Patensie, and Hankey). The samples from the Bonnievale and De Hoop localities were preserved directly in 100% ethanol; hence, no allozyme data are available for these two localities. Populations from the lower Olifants River system were not included because they belong to a separate, distinct species (Daniels *et al*., 1999a). Crabs were caught with

Table 1. Enzymes and buffer systems used during electrophoresis. *n* is the number of loci examined, A, B, and C are the buffer systems used. Detailed of these can be obtained from Daniels *et al*., (2002). E. C. = Enzyme Commission numbers.

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*	A	1.2.1.12	1
Isocitric dehydrogenase	*Idh*	B	1.1.1.42	2
Lactate dehydrogenase	*Ldh*	B	1.1.1.27	1
Peptidase (Glycyl leucine as substrate)	*Gl*	B	3.4.11 -	1
Peptidase (Leucine tyrosine as substrate)	*Lt*	A	3.4.11 -	2
Malate dehydrogenase	*Mdh*	C	1.1.1.37	2
Malic enzyme	*Me*	C	1.1.1.40	1
Mannose phosphate isomerase	*Mpi*	C	5.3.1.8	1
Phosphoglucomutase isomerase	*Pgm*	A	2.7.5.1	2

handnets following their attraction to baited (ox heart) lines. On capture, live crabs were transferred to the laboratory and killed by freezing at –20° or –80°C for 24 h prior to tissue extraction.

Allozymes

Muscle tissue was removed from each specimen, and tissue samples were placed in cryotubes and stored in liquid nitrogen. The genetic variation at 15 allozyme loci, coding for 11 enzymes, was consistently scored for all the populations (Table 1). The protocol for staining can be found in Daniels *et al*. (1999a). Numerical analyses were performed using the BIOSYS-1 program (Swofford and Selander, 1981). The mean heterozygosity per locus (H_o) and expected heterozygosity (H_e) for each population were 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. The mean unbiased genetic identities (I) among the populations were calculated from the allelic frequencies according to Nei (1978).

We calculated Wright's (1965) F statistics, F_{ST} (the genetic differentiation between subpopulations), to determine the degree of genetic differentiation amongst the populations using the statistical package FSTAT (Goudet, 2000). The degree of genetic structuring was calculated over all the populations. In addition, we used GENEPOP (version 1.2) (Raymond and Rousset, 1995) to determine the level of gene flow (number of migrants = Nm) within drainages (Olifants, Berg, Breë, and Gamtoos Rivers) and over all the populations among all drainages, using the private allele method.

Sequencing

DNA was extracted from three to four individuals from 10 representative populations (Citrusdal, Clanwilliam, Paarl, Tokai, Bonnievale, De Hoop, Robertson, Tradouwspass, Kleinplaats, and Poortjies) that span the distribution covered by the allozyme data set. The same muscle tissue stored at –80°C used in the allozyme study was also used for DNA extraction. Total genomic DNA was isolated from 0.05 g of muscle tissue. The tissue was digested in 500 µL of DNA lysis buffer (200 mL of 1× STE–100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA – and 30 mL of 20% SDS solution) 20 µL proteinase K and 10 µL RNAase. This mixture was incubated for 2–3 hours at 55°C followed by a DNA extraction using the standard phenol/chloroform : isopropanol protocol.

The two primer sets, 16S (16S ar and 16S br, Cunningham *et al*., 1992) and COI (LCO-1490 and HCO-2198, Folmer *et al*., 1994) were used to amplify each of the two gene regions using standard PCR methods. For a detailed outline, see Daniels *et al*. (2002b). The 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 primer dilution. Unincorporated dideoxynucleotides were removed by matrix filtration using Sepadex G-25 (Sigma). This was followed by analysis on an ABI 3100 automated DNA sequencer.

Each sample was checked for base ambiguity in Sequence Navigator (Applied Biosystems). The 16S rRNA sequences were aligned in CLUSTAL X version 1.81 (Thompson *et al*., 1997), 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. The COI sequences were aligned manually.

Phylogenetic analyses were performed in PAUP*4 version beta 10 (Swofford, 2001) using neighbor joining (NJ), parsimony (MP), and maximum likelihood (ML) methods. For the MP analysis, trees were generated using the heuristic search option with TBR branch swapping and 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 (Posada and Crandall, 2001; Whelan *et al*., 2001). For the ML analysis, MODELTEST version 3.06 (Posada and Crandall, 1998) was used to determine the best-fit substitution model. For NJ, the best-fit ML model with the appropriate gamma correction obtained from MODELTEST was used to calculate genetic distances and construct the NJ tree. Statistical support for the nodes was estimated by bootstrapping replicates (Felsenstein, 1985). For MP and NJ, 1000 bootstrap replicates were performed, whereas for ML, 100 bootstrap replicates were performed due to computational constraints. In this paper, bootstrap values less than 50% are considered unsupported, bootstrap values between 50% and 70% as weakly supported, and values greater than 70% as strongly supported (Baum *et al*., 1998; Graham *et al*., 1999). All trees were rooted using *Potamonautes unispinus* as an outgroup. Outgroup selection was based on a molecular

Table 2. The mean number of alleles per locus, the percentage of the loci that were polymorphic, and the observed and expected heterozygosity (H_o and H_e, respectively) obtained from the allozyme data.

Population	Mean sample size	Mean no. of alleles	Percentage polymorphic loci	H_o	H_e
Citrusdal	10.0	1.0	0.0	0.000	0.000
Boontjieskraal	18.7	1.1	6.7	0.004	0.004
Kriedouwkrans	16.4	1.0	0.0	0.000	0.000
Clanwilliam	13.8	1.1	6.7	0.013	0.013
Paarl	18.9	1.2	13.3	0.022	0.021
Tunnel Terminals	22.4	1.1	6.7	0.006	0.006
Water Station	20.0	1.0	0.0	0.000	0.000
Liesbeek	9.7	1.1	6.7	0.013	0.013
Tokai	22.0	1.3	20.0	0.035	0.043
Stellenbosch	24.5	1.3	13.3	0.025	0.050
Bainskloof	15.0	1.0	0.0	0.000	0.000
Robertson	10.0	1.0	0.0	0.000	0.000
Tradouws Pass	27.4	1.5	20.0	0.028	0.065
Tsitsikamma	9.7	1.0	0.0	0.000	0.000
Sandriver	10.0	1.0	0.0	0.000	0.000
Bosdorp	26.3	1.1	13.3	0.009	0.000
Smithskraal	15.3	1.3	20.0	0.030	0.035
Kleinplaats	20.0	1.2	20.0	0.033	0.030
Poortjies	21	1.1	6.7	0.006	0.006
Patensie	17.3	1.3	20.0	0.031	0.054
Hankey	25.0	1.1	6.7	0.003	0.003

phylogeny for *Potamonautes*, where *P. unispinus* forms a well-supported monophyletic clade with other large-bodied freshwater crab species, including *P. perlatus* (Daniels *et al*., 2002b). The two mtDNA data sets were combined, for mitochondrial genes are linked as an effectively single locus. The best-fit substitution model was again determined using MODELTEST. The Shimodaira and Hasegawa (1999) test as implemented in PAUP*4 version beta 10 was used on the total evidence tree to enforce constrained relationships. Population subdivision was estimated using an Analysis of Molecular Variance (AMOVA) as implemented in ARLEQUIN 2.0 (Schneider *et al*., 1997); among populations variation was F_{SC}, within populations was F_{ST}, and among drainages variation was F_{CT}. Nucleotide (π) and halotype diversity (δ) was calculated using the method outlined by Nei (1987).

In an attempt to apply a molecular clock to the data, a mutation rate of 1% per million years was used for 16S, derived from Schubart *et al*. (1998), whereas a mutation rate of 2.3% was used for COI based on studies by Knowlton *et al*. (1993) and Brower (1994). These mutation rates were calculated for crustaceans and butterflies. More recently, Ketmaier *et al*. (2003) demonstrated that the COI rates appear very constant in aquatic isopods, lending additional support to the COI molecular clock. However, it is noteworthy that molecular clocks are not ideal and may indeed be highly variable between different lineages. In the present study, these dates are applied with caution, particularly in the absence of fossil data for the freshwater crabs.

Results

Allozymes

Of the 15 loci examined, nine loci were monomorphic (*Ark-1*, *Gap-1*, *Me-1*, *Mdh-1*, *Lt-2*, *Ldh-1*, *Gl-1*, *Mpi-1*, and *Pgm-2*), whereas the remaining six loci were polymorphic (*Idh-1*, *Idh-2*, *Mdh-2*, *Lt-1*, *Gpi-1*, and *Pgm-1*). The allele frequencies for the six polymorphic loci are presented in Appendix I. For the six polymorphic loci, the number of alleles ranged from two in *Idh-1* and *Idh-2*, three in *Mdh-2* and *Lt-1*, four in *Gpi-1*, to five alleles in *Pgm-1*. No single locus was polymorphic in all the populations.

The allele frequencies were used to determine the degree of genetic similarity (I) between the populations. The mean genetic similarity between all the populations was $I = 0.98$. No macro-geographic pattern could be detected using genetic I values; hence, no UPGMA was calculated. The mean number of alleles ranged from 1.0 to 1.5 between populations, whereas the percentage of polymorphic loci ranged from 0.0% to 20%. In addition, the expected heterozygosity ranged from 0.0 to 0.065, and the observed heterozygosity varied from 0.0 to 0.035 (Table 2).

Within the Berg River system, the mean F_{ST} was 0.039, and the two polymorphic loci were *Gpi-1* and *Mdh-2*, where the F_{ST} values were 0.018 and 0.057, respectively. In the Breë River system, the mean F_{ST} was 0.296, and the three polymorphic loci were *Gpi-1*, *Mdh-1*, and *Pgm-1*, where the F_{ST} values were 0.082, 0.022, and 0.366, respectively. Within the Olifants River system, the mean F_{ST} was 0.065, and the two polymorphic loci were *Gpi-1* and *Mdh-2*, where the F_{ST} values were 0.020 and 0.077,

respectively. In the Gamtoos River, the mean F_{ST} was 0.206, clearly indicating genetic substructuring. Within this drainage system, four loci were polymorphic; these were *Idh-1* and *-2*, *Gpi-1*, and *Pgm-1*. The F_{ST} values ranged from 0.017 to 0.289 in *Idh-2* and *Pgm-1*, respectively.

Over all loci and all the populations, the mean F_{ST} was 0.214. In the Olifants River, no significant population structuring was evident (Appendix II). The wide-scale absence of significant genetic structure between populations suggests that in the recent past, all these populations were part of the same interbreeding population. The levels of gene flow (Number of migrants) within drainage systems were always greater than 1 (Olifants River, $Nm = 2.5$; Berg River, $Nm = 3.2$; Breë River, $Nm = 2.0$; Gamtoos River, $Nm = 2.3$), whereas over all populations, the Nm was 1.7. These results indicate that levels of gene flow between populations are probably high, resulting in the homogenization of gene pools.

16S Sequence Data

A 400 base pair fragment of the 16S rRNA mtDNA gene was amplified and sequenced from 37 specimens from 10 populations. Sequences have been deposited in GenBank (Acc. Numbers AF 493160–493176). Compositional bias with unequal proportions of the bases is characteristic in most mtDNA studies. All three phylogenetic methods retrieved the same topology. For the MP analysis, 15 informative characters (CI = 0.76; RI = 0.95) and a tree length of 21 steps were found. A strict consensus of 210 equally parsimonious trees recovered two distinct clades, similar to those recovered by ML and NJ. For the ML tree (–lnL = 838.54), the HKY-85 model (Hasegawa *et al*., 1985) was selected using MODELTEST and a transition/ transversion ratio (ti/tv) of 1.10 with the base frequencies A = 37.05%, C = 9.89%, G = 17.64%, and T = 35.42%. A bootstrapped ML tree of the sequence data retrieved two distinct clades (Fig. 2). Clade 1 (supported with 81%) was composed of all the river systems that drained the western escarpment and was composed of the populations from the Olifants (Citrusdal and Clanwilliam) and Berg River (Paarl), together with the Tokai population. Clade 2, recovered with 94% bootstrap support, was composed of populations sampled from the Gamtoos River system (Kleinplaats and Poortjies) (the yellow colored crabs) forming a single clade with populations sampled from the Breë River system (Bonnievale, Robertson, and Bainskloof) and De Hoop. These latter two rivers (Gamtoos and Breë Rivers) drain the southern part of the escarpment. Bootstrap support for clades within the latter two drainages was low.

Of the 37 specimens sequenced, a total of 17 haplotypes were identified. The number of haplotypes within a single population ranged from one to four. The same haplotype that occurred in the Olifants River system in the Clanwilliam population was also present at low frequency in the Paarl population, providing possible evidence for interdrainage dispersal, or it may be a retained ancestral haplotype. Similarly, a haplotype found in the Robertson population was present in the Bonnievale population. Most of the unique haplotypes differed by a number of base pairs. The AMOVA indicated that among populations within drainages, 38.30% variation was present ($F_{SC} = 0.4542$; $P < 0.01$), whereas 46.01% variation ($F_{ST} = 0.5398$; $P < 0.01$) occurred within populations. Among drainages, 15.68% variation ($F_{CT} = 0.1568$; $P < 0.01$) was evident. The nucleotide diversity (π) ranged from 0 to 0.089, whereas the haplotype diversity (δ) ranged from 0 to 1 (Table 3).

The corrected sequence divergence values within Clade 1 were 1% and in Clade 2 were 4%, whereas the maximum divergence between the two clades was 6%. The application of a molecular clock places the divergence of the two clades in the Pliocene, 5 million years before present (MYBP).

COI Sequence Data

A 413 base pair fragment of the COI gene was amplified and sequenced from 37 specimens from 10 populations. Sequences have been deposited in GenBank (Acc. No. AF 494022–494036). All three phylogenetic methods recovered the same topology. The MP analysis found 18 informative characters (CI = 0.85; RI = 0.97) and a tree length of 21 steps. A strict consensus of 39 equally parsimonious trees recovered one distinct clade comprised of the populations in clade one as retrieved with the 16S data. For ML (–lnL = 979.63), the HKY-85 model was selected using MODELTEST, with the base frequencies A = 35.88%, C = 13.33%, G = 21.52%, and T = 29.21% and a ti/tv ratio of 4.28 and a gamma ($\alpha = 0.014$) selected for the analysis. The tree derived from ML analysis of the COI sequence data was

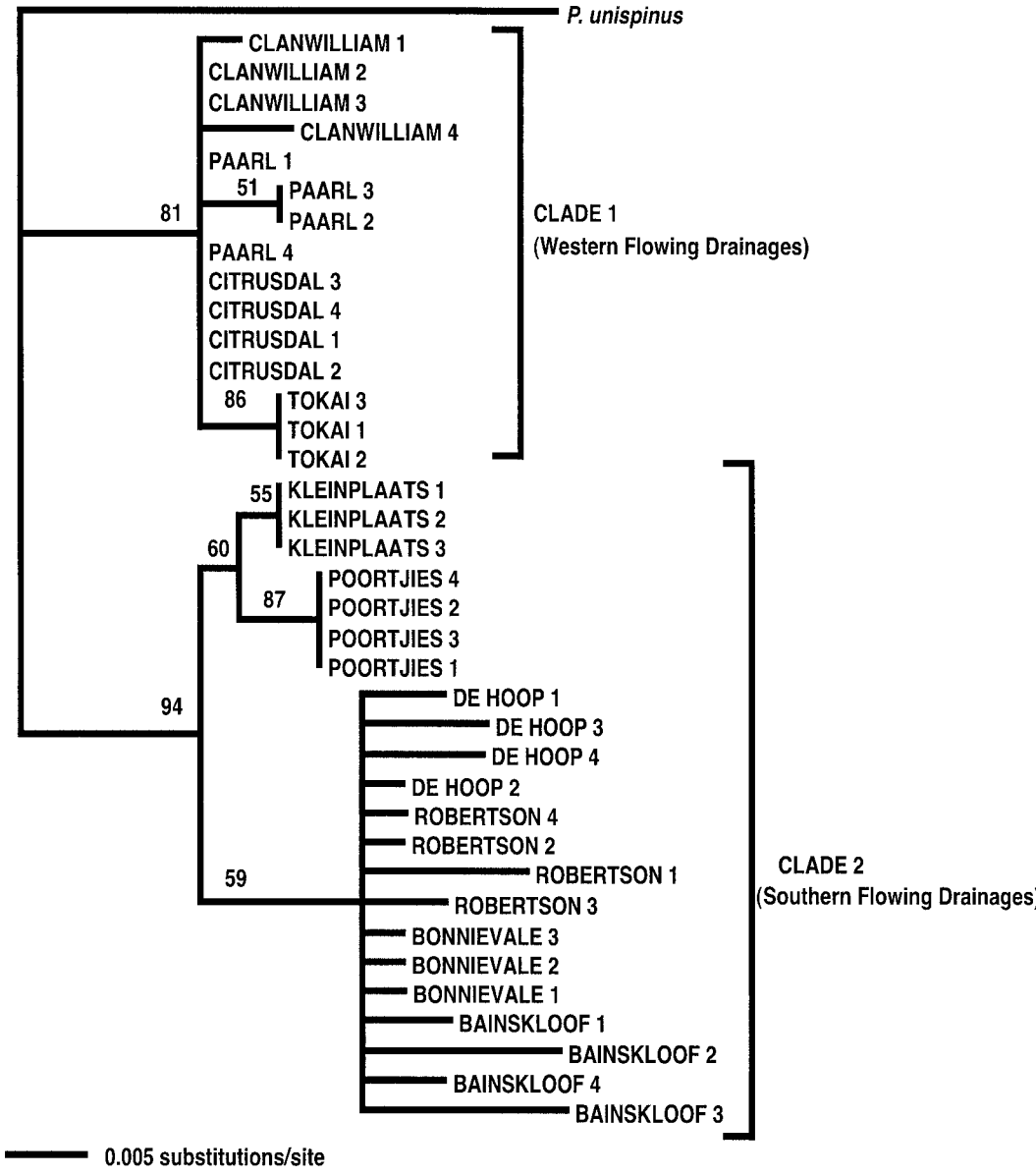

Fig. 2. A bootstrapped ML tree (–lnL = 838.54) based on the 16S rRNA mt DNA data set for the 10 populations sampled. Bootstrap values less than 50% are not shown; branch lengths are shown on the tree.

highly congruent to the 16S topology. A bootstrapped ML tree is shown (Fig. 3). Similar to the 16S data set, Clade 1 was composed of all the river systems that drained the western escarpment, and included the Olifants and Berg River samples, together with the Tokai population. Within this clade, the close phylogenetic relationship between the Berg and Olifants rivers and the Tokai population is well supported (71%). In contrast to the 16S data set, a second well-supported clade (with bootstrap values greater than 75%) was absent.

Of the 37 specimens sequenced, a total of 16 haplotypes were identified. The number of haplotypes within a single population ranged from one to four. Within certain drainages, for example in the Olifants River, a single haplotype was present. Most of the unique haplotypes differed by a number of base pairs. The AMOVA indicated that 38.07% variation

Table 3. Haplotype (δ) and nucleotide diversity (π) with 95% confidence intervals, for the freshwater crab populations sampled for the two gene regions. Where the haplotype or nucleotide diversity is zero, no confidence intervals are given.

	Haplotype diversity (δ)		Nucleotide diversity (π)	
Population	16S	COI	16S	COI
Citrusdal	0	0	0	0
Clanwilliam	0.833 (± 0.222)	0	0.0089 (± 0.006)	0
Tokai	0	0.667 (± 0.314)	0	0.001 (± 0.002)
Bainskloof	1.00 (± 0.176)	0.833 (± 0.222)	0.025 (± 0.017)	0.011 (± 0.008)
Bonnievale	0	0.666 (± 0.314)	0	0.005 (± 0.004)
De Hoop	0.833 (± 0.222)	0.500 (± 0.265)	0.007 (± 0.005)	0.001 (± 0.001)
Paarl	0.667 (± 0.204)	0	0.005 (± 0.004)	0
Robertson	0.833 (± 0.222)	0.833 (± 0.222)	0.002 (± 0.002)	0.012 (± 0.009)
Poortjies	0	0.500 (± 0.265)	0	0.001 (± 0.001)
Kleinplaats	0	0	0	0

($F_{SC} = 0.4895$; $P < 0.01$) was occurring among populations within drainages. In addition, within populations 39.69% variation ($F_{ST} = 0.6031$; $P < 0.01$) was present. Among drainages, 22.24% of variation ($F_{CT} = 0.2224$; $P < 0.01$) was evident. The nucleotide diversity (π) ranged from 0 to 0.012, whereas the haplotype diversity (δ) ranged from 0 to 0.833 (Table 3).

On average, the sequence divergence between clades was twice as high with COI compared to 16S distances. Within Clade 1, the maximum corrected sequence divergence was 3%; within Clade 2 the maximum corrected sequence divergence was 6%, and the overall sequence divergence was 10%. The application of a molecular clock to our data indicates that most of the divergence has occurred since the Pliocene.

Combined Analysis

The parsimony analysis of the combined data set found 31 informative characters and yielded 44 trees that were 44 steps long (CI = 0.75; RI = 0.95). A consensus tree was created that had the same topology as the COI tree, while a MP bootstrapped tree supported the same nodes as the ML tree. For ML (−1nL = 2071.12; Fig. 4) the TrN+Γ model (Rodriguez *et al*., 1990) was selected using MODELTEST ($R_1 = R_3 = R_4 = R_6 = 1$; $R_2 = 6.64$; $R_5 = 3.43$), with the base frequencies at A = 36.40%, C = 11.97%, G = 18.78%, and T = 32.85%; the α was 0.1206. The ML bootstrapped topology was similar to the COI tree. Briefly, only a single well-supported clade was consistently recovered (previously called Clade 1) (72% bootstrap support) that comprised all the populations sampled from the westerly-flowing drainages (Citrusdal, Clanwilliam, Tokai, and Paarl). The samples from the Gamtoos River fell into a well-supported clade (98%). However, unlike in the 16S data set, it was not closely related to the populations from the Breë River drainage system (southern-flowing drainage). A constrained analysis using the Shimodaira and Hasegawa (1999) test to enforce the monophyly between the Gamtoon River populations and those in Clade 1 (Olifants, Berg River, and Tokai) recovered a significantly worse topology when compared to the best tree (Δ−1nL = 292.67; $P < 0.001$). In addition, when the Gamtoos populations were forced to be monophyletic with the Breë River drainage, a significantly worse topology was obtained (Δ−1nL = 207.05; $P < 0.001$).

Discussion

Contrasting patterns of genetic variation were observed between the allozyme and sequence data. While the allozyme data demonstrated genetic invariance between the sampled populations, the sequence data revealed highly distinct clades that differentiated populations of *Potamonautes perlatus* that occur in western and southern drainage systems. Collectively, these results demonstrate that sequence data have considerably superior resolution power compared to allozyme data in detecting population structuring and that inferences of population genetic homogeneity derived from allozyme invariance should be interpreted with caution. However, at a small spatial scale, within drainage systems, for example, both the allozymes and sequence data indicate the presence of high levels of gene flow as evident from the general absence of genetic variation. The data further indicate that the yellow specimens that occur in the Gamtoos River are

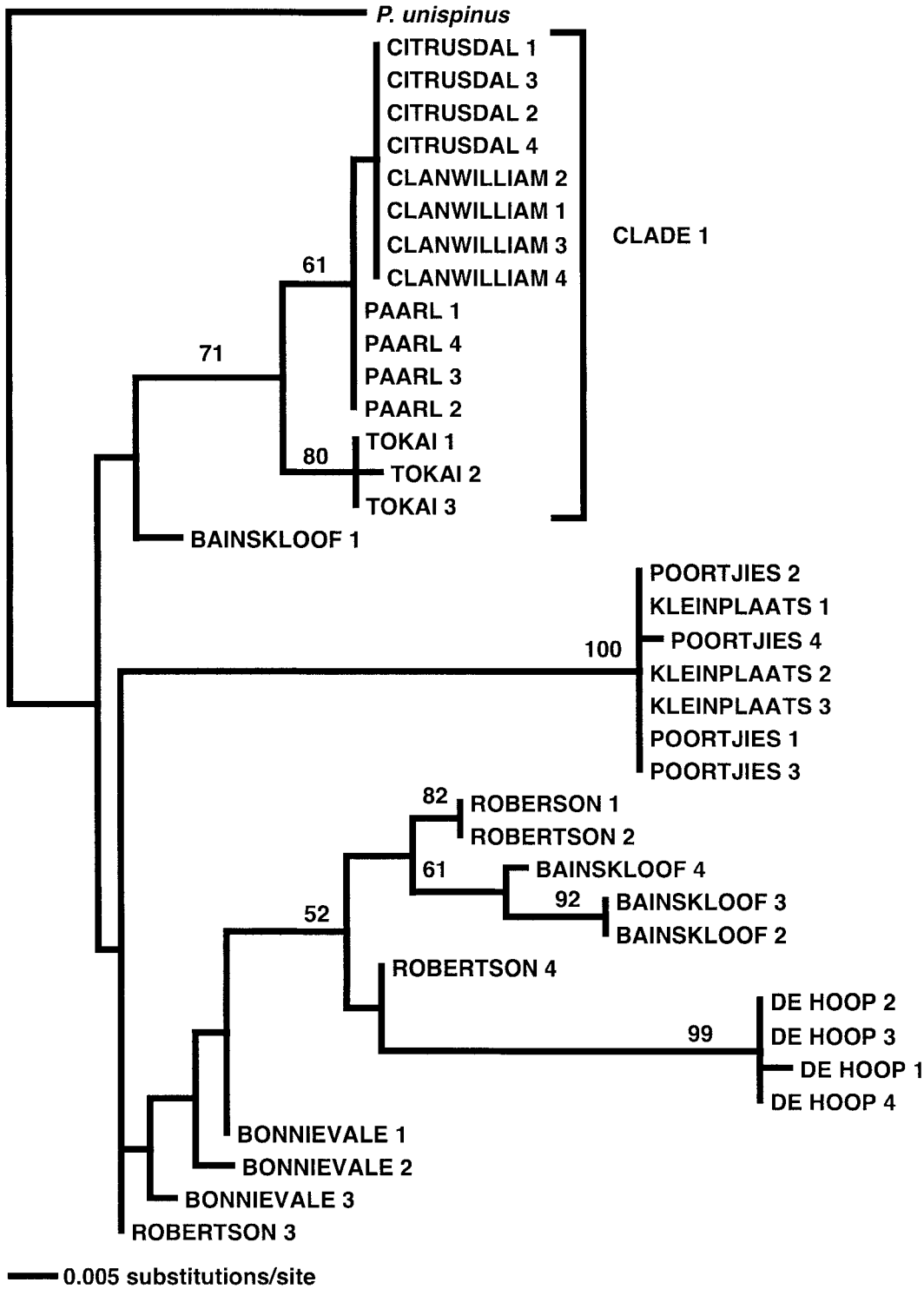

Fig. 3. A bootstrapped ML tree (–lnL = 979.63) based on the COI mt data set for the 10 populations sampled. Bootstrap values less than 50% are not shown; branch lengths are shown on the tree.

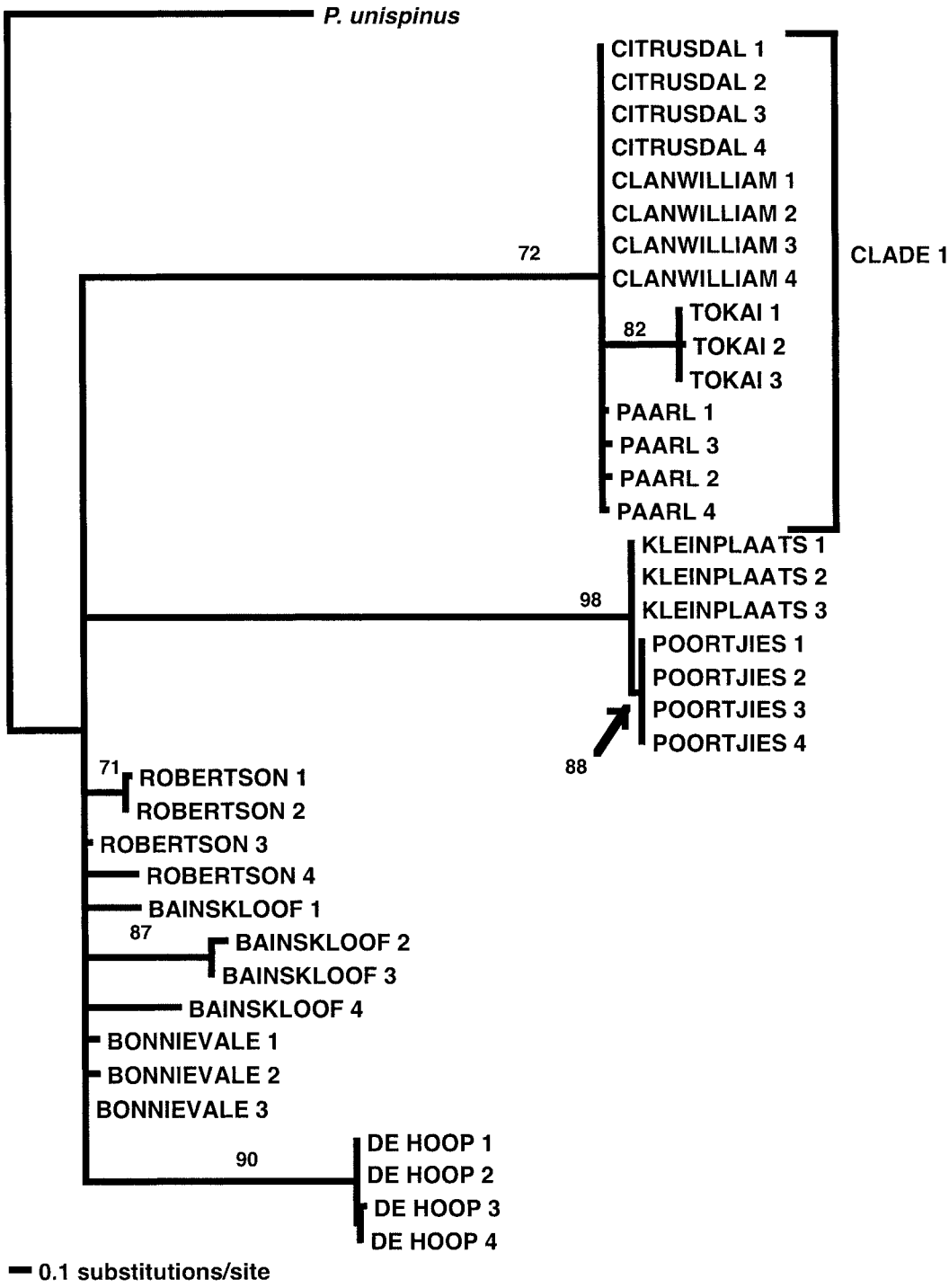

Fig. 4. A combined ML tree (–lnL = 2071.12) based on the 16S rRNA and COI mt genes for the 10 populations sampled. Bootstrap values less than 50% are not shown; branch lengths are shown on the tree.

Table 4. Intraspecific levels of genetic differentiation as determined by allozyme electrophoresis for the southern African river crab species (*Potamonautes* spp.) studied to date (number of populations = *n* pop; number of loci screened = *n* loci; mean number of alleles per locus = *n* alleles; mean percentage of loci polymorphic = % polymorphic loci; mean observed heterozygosity = H_o, and genetic identity = *I*). The results presented here are from a number of published studies. The numbers at the beginning of each species provide the reference to the study.

Species	*n* pop	*n* loci	*n* alleles	Percentage polymorphic loci	H_o	*I*
1) *P. brincki*	6	13	1.12	7.70	0.006	>0.91
2) *P. clarus*	3	21	1.10	12.70	0.020	>0.99
3) *P. calcaratus*	5	21	1.10	5.74	0.020	>0.99
2) *P. depressus*	2	21	1.30	21.70	0.025	>0.99
4) *P. lividus*	3	21	1.26	20.63	0.038	>0.94
5) *P. granularis*	5	14	1.26	17.14	0.037	>0.99
1) *P. parvicorpus*	4	13	1.20	19.25	0.037	>0.86
6) *P. parvispina*	10	14	1.27	11.39	0.015	>0.99
7) *P. perlatus*	21	15	1.13	8.57	0.012	>0.99
8) *P. sidenyi*	31	11	1.27	28.21	0.065	>0.74
9) *P. unispinus*	18	17	1.15	10.79	0.023	>0.94
9) *P. warreni*	10	17	1.10	7.66	0.010	>0.94

1) Daniels *et al*., (2001a); 2) Daniels *et al*., (2003); 3) Daniels *et al*., (2002a); 4) Gouws *et al*., (2001); 5) Daniels *et al*., (1999); 6) Daniels *et al*., (1998a); 7) present study; 8) Gouws *et al*., (2002); and 9) Daniels *et al*., (2001b).

conspecific and do not represent a distinct taxon, as genetic differentiation between this river system was low compared to those observed in other studies (Daniel *et al*., 2001a, b). The high genetic *I* values greater than 0.98 and the absence of fixed allelic differences indicate that the Gamtoos populations are conspecific with other *Potamonautes perlatus* populations. The occurrence of color variation is thus possibly a result of eco-phenotypically induced factors or strong selection, and does not appear to have a genetic basis that can be detected with the use of allozymes. Similarly, Okano *et al*. (2000) recently reported the absence of genetic variation among color forms of *Geothelphusa dehaani* from the Japanese mainland. Although color has previously been used as a diagnostic character for freshwater crab species, color may be variable, particularly in taxa that are widespread, such as *P. perlatus*.

Allozymes

A summary of all the allozyme studies of southern African freshwater crabs is presented in Table 4. Genetic variation within all of the freshwater crab species is generally low. For example, intraspecific genetic *I* values range from 0.91 to 1.00 for most taxa, except for *P. sidneyi*—the latter taxon may be composed of genetically distinct populations characterized by a number of fixed allele differences (Gouws *et al*., 2002). The mean observed heterozygosity for all freshwater crabs were low and ranged from 0.006 to 0.065, these values compare favorably with those observed in a number of other crustacean species (Hedgecock *et al*., 1982). Similarly, the low level of polymorphism reported in the present study (ranging from 7.66% to 21.70%) compares well with that observed for other freshwater crabs (Table 2). Genetic variation within freshwater crabs as shown by allozymes is thus clearly low.

The genetic variation across all the *P. perlatus* populations was pronounced (F_{ST} = 0.21). These values are generally higher than those reported for conspecific mountain stream invertebrate taxa and generally more similar to those reported for riverine and semiterrestrial species. For example, F_{ST} values in mountain stream taxa were 0.032, 0.085, and 0.127 in *P. parvispina*, *P. clarus*, and *P. depressus*, respectively, whereas F_{ST} values among populations of *P. parvicorpus* and *P. brincki* were 0.485 and 0.085, respectively (Daniels, unpublished data; Daniels *et al*., 2001a). Among riverine species, Daniels *et al*. (2001b) reported a mean F_{ST} values of 0.417 and 0.690 for *P. warreni* and *P. unispinus*, respectively. More recently, Gouws *et al*. (2002) reported an F_{ST} value of 0.534 for allopatric populations of *P. sidneyi*. Among the semiterrestrial species *P. lividus* and *P. calcaratus*, the mean F_{ST} values were 0.314 to 0.125, respectively. Generally, similar F_{ST} values have been reported for a number of other freshwater crustaceans (Hughes *et al*., 1995, 1996). For example, in the aquatic atyid species *Paratya australiensis*, Hughes *et al*. (1995) reported that within streams, the F_{ST} value was 0.006, whereas between streams, the mean value was 0.57. In

their study, gene flow was largely restricted to within the catchment. This is also evident within and between all drainages sampled in the present study, as sufficient gene exchange ($Nm > 1$) between populations is occurring to prevent genetic structuring. However, these results may simply reflect the inability of this marker (allozymes) to reliably detect gene flow levels.

The high F_{ST} values reported in the present study corroborate the fact that *P. perlatus* probably has limited capacity to disperse over large geographic areas. Hurwood and Hughes (2001) recently reported that the freshwater shrimp *Caridina zebra* (a species less than 2 cm in length) is capable of terrestrial dispersal. Similarly, in the Australian atyid shrimp *Paratya australiensis*, clear upstream movements have been reported. The present study provides corroborative evidence that freshwater crabs (with a mean carapace length of 29 mm for 1149 individuals) may have the potential for dispersing short distances across land (Daniels, unpublished data). Dispersal events may typically occur during flooding or conditions of high humidity. Freshwater crab species are well known to be amphibious (Barbaresi *et al.*, 1997). However, it remains unknown how far *P. perlatus* can move during periods of active dispersal. Field studies are needed to complement the levels of gene flow observed.

Sequencing

The sequence data were largely congruent, with both gene fragments detecting essentially the same patterns of population structure. Two major clades composed of westward and southward flowing drainages were evident from the sequence data. Interestingly, drainages that share the same mountainous regions as areas of origin (Berg and Olifants rivers) had very low levels of sequence divergence between populations. For example, the sequence divergence between the Berg and Olifants river systems based on the COI gene fragment was 0.2%. These results are corroborated by an allozyme study on a mountain stream freshwater crab, *P. parvicorpus*, where low levels of genetic variance were detected among populations from the Berg and Olifants rivers (Daniels *et al.*, 1998). Similar results are evident within each of the two clades, indicating that dispersal may be occurring. The maximum intraspecific sequence divergence (6% with 16S and 10% with COI) between *P. perlatus* populations is higher than values reported for interspecific comparisons for other decapod crab species. For example, between *P. sidneyi* and *P. perlatus*, the sequence divergence for 16S is 3.5%, whereas between *P. sidneyi* and *P. unispinus*, the sequence divergence is 4.5% (Daniels, unpublished data). Geller *et al.* (1997) reported sequence divergence values of 2.1% between two stone crab *Menippe* species, whereas between shore crab *Carcinus* species, sequence divergence values ranged 2.5–3.9%. For the COI fragment, sequence divergence between the species pairs *P. perlatus* and *P. warreni*; *P. sidneyi* and *P. unispinus* was nearly 8% (Daniels, unpublished data). The clear geographic division between the westward and eastward flowing drainage systems indicates historic isolation between populations from these rivers. The application of a molecular clock for both the 16S and COI indicates that the time of divergence between the two major clades probably occurred during the early Pliocene (6 MYBP) (Deacon, 1985; Tyson, 1986).

River systems frequently change course through river capture and would allow for the dispersal of freshwater animals that have been previously isolated. Little is known about drainage capture of southern African river systems. Certainly, one of the most interesting aspects of the present study is the fact that even though some populations were collected in relatively close geographic proximity (for example Paarl–Berg river system and Bainskloof–Breë river system), they fell into either of these two clades, with the most common delimiting factor being the Cape Fold mountain range (Fig. 1). Tectonic activity in this area has occurred during the Miocene, causing major uplifts throughout South Africa. After a period of quiescence, vast tectonic upheavals were reinitiated in the late Pliocene, raising interior plateaus by 1000 m (King, 1978). The mountain ranges thus appear to be an important factor that sculpted the genetic differentiation between the two main drainage clades (western and southern flowing). It is thus likely that the cumulative change in geology and climate are the most likely mechanisms to have aided in the formation of these two distinct clades.

Besides the study on the freshwater crab *P. parvicorpus*, an endemic to the Berg and Olifants rivers, no comparative genetic studies on other species exist that have sampled the same geographic areas as in the present study. However, what is interesting is that freshwater

fishes that are incapable of navigating drainages have undergone pronounced divergence between the western and southern mountain regions. An intraspecific study on the Cape galaxid fish (*Galaxias zebratus*) undertaken by Waters and Cambray (1997) revealed that populations of this freshwater fish in drainage systems on the west coast were indeed genetically distinct from those on the south and east coast. These authors suspect that the divergence between these drainages occurred during the early Pliocene.

Conclusion

Although allozyme data may be invaluable for providing estimates of gene flow on a small spatial scale and for detecting distinct gene pools, it provides limited resolution in detecting population genetic structure at large spatial scales. Sequence data, on the other hand, can be used with more reliability to detect isolation between conspecific populations and should provide significant insight into factors that have sculpted contemporary distribution patterns.

In the present study, the sequence data detected two distinct and highly divergent clades. The lack of resolution with the allozyme data could probably be attributed to the fact that allozymes are slower evolving molecules; in contrast, the high discriminatory power of the mitochondrial sequence data could probably be attributed to its smaller effective population size and high mutation rate. Although the sequence divergence between populations is marked, the genetically distinct clades cannot be regarded as distinct taxa, as no fixed morphological difference and nuclear (allozyme) differences were observed between the sampled populations. At least three distinct management units, as defined by Moritz (1994), can be identified from this study: populations in Clade 1 (Olifants and Berg rivers and Tokai), the Breë River populations, and the Gamtoos River population.

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Appendix I. Allele frequencies for the six polymorphic loci over the 21 *Potamonautes perlatus* populations sampled. The population numbers designated below are identical to those localities indicated on the map (fig. 1). n = sample size.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Allele	Locus: *Idh-1*																				
n	10	19	18	10	19	23	20	10	23	25	15	10	30	10	10	28	16	20	21	18	25
B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.944	1
D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.056	0
	Locus: *Idh-2*																				
n	10	19	18	10	19	23	20	10	23	25	15	10	30	10	10	28	16	20	21	18	25
B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.982	0.969	0.975	0.952	1	1
D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.018	0.031	0.025	0.048	0	0
	Locus: *Lt-1*																				
n	10	19	11	10	19	23	20	10	20	25	15	10	25	5	10	28	16	20	21	18	25
A	0	0	0	0	0	0	0	0	0.15	0	0	0	0	0	0	0	0	0	0	0	0
B	1	1	1	1	1	1	1	1	0.8	1	1	1	1	1	1	1	1	1	1	1	1
D	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0
	Locus: *Gpi-1*																				
n	10	19	23	20	19	23	20	10	30	22	15	10	30	10	10	28	15	20	21	18	25
B	1	1	1	1	0.947	0.957	1	0.9	0.883	0.682	1	1	0.867	1	1	1	0.9	0.925	1	0.944	0.98
C	0	0	0	0	0	0.022	0	0.1	0.1	0.045	0	0	0.117	0	0	0	0	0	0	0	0
D	0	0	0	0	0.053	0.022	0	0	0.017	0	0	0	0	0	0	0	0.1	0.075	0	0.056	0.02
H	0	0	0	0	0	0	0	0	0	0.273	0	0	0.017	0	0	0	0	0	0	0	0
	Locus: *Mdh-2*																				
n	10	19	11	10	18	23	20	10	23	25	15	10	30	10	10	28	16	20	21	18	25
A	1	0.974	1	1	0.889	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
B	0	0.026	0	0	0.056	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0.056	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Locus: *Pgm-1*																				
n	10	19	16	10	19	23	20	10	23	20	15	10	27	10	10	19	16	20	21	16	25
A	0	0	0	0.1	0	0	0	0	0.043	0.075	0	0	0.426	0	0	0.053	0.031	0.15	0	0.5	0
B	1	1	1	0.9	1	1	1	1	0.957	0.85	1	1	0.389	1	1	0.947	0.844	0.85	1	0.406	1
C	0	0	0	0	0	0	0	0	0	0.05	0	0	0.074	0	0	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0	0	0.025	0	0	0.074	0	0	0	0.125	0	0	0.094	0
F	0	0	0	0	0	0	0	0	0	0	0	0	0.037	0	0	0	0	0	0	0	0

Appendix II. Pairwise F_{ST} values over all populations. Values that are bolded are not statistically significant, whereas the remaining values are significant statistically.

	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	**−0.0189**	–	**0.0556**	**0.0217**	**−0.0125**	–	**0.0556**	**0.0555**	**0.1269**	–	–	0.2771	–	–	**−0.0037**	**0.0464**	**0.0572**	**−0.0006**	0.3528	**−0.0219**
2		**−0.0158**	**0.0669**	**0.0203**	0.0059	0.0014	**0.0669**	**0.0873**	0.1754	**−0.0065**	**−0.0189**	**0.3305**	**−0.0189**	**−0.0189**	**0.0161**	**0.0802**	0.0833	**0.0152**	0.4155	**0.0009**
3			**0.0922**	0.0351	0.0114	–	**0.1268**	**0.0735**	0.1881	–	–	**0.3300**	–	–	**0.0131**	**0.0857**	0.0888	0.0197	0.4202	−0.0017
4				0.0515	0.0768	0.1126	0.0790	**0.0565**	0.1222	0.0867	0.0556	0.1979	0.0556	0.0556	−0.0202	0.0180	−0.0058	0.0604	0.2412	0.0985
5					0.0299	0.0531	0.0255	**0.0729**	0.1328	0.0396	0.0217	0.2983	0.0217	0.0217	0.0521	0.0473	0.0589	0.0472	0.3562	0.0427
6						0.0076	0.0075	**0.0792**	0.1609	−0.0002	−0.0125	0.3381	−0.0125	−0.0125	0.0214	0.0646	0.0718	0.0186	0.4319	−0.0126
7							0.1126	**0.0956**	0.1884	–	–	0.3442	–	–	**0.0194**	0.0933	0.0947	0.0233	0.4456	−0.0046
8								**0.0266**	**0.0756**	**0.0867**	0.0556	0.2518	**0.0556**	0.0556	**0.0540**	**0.0314**	0.0488	0.0604	0.3273	0.0651
9									0.1101	**0.0782**	0.0555	0.2423	**0.0266**	**0.0555**	**0.0899**	**0.0605**	0.0654	0.0911	0.2855	0.0989
10										0.1610	0.1269	0.1867	**0.1269**	**0.1269**	0.1633	0.0692	0.0851	0.1796	0.2346	0.1913
11											–	0.3138	–	–	**0.0100**	**0.0726**	**0.0780**	0.0135	0.4039	−0.0110
12												**0.2771**	–	–	**−0.0037**	**0.0464**	**0.0572**	0.0006	0.3528	−0.0219
13													**0.2771**	0.2771	**0.2820**	**0.1800**	0.1629	0.3352	−0.0141	0.3601
14														–	**−0.0037**	**0.0464**	0.0572	0.0006	**0.3528**	−0.0219
15															**−0.0037**	**0.0464**	0.0572	−0.0006	**0.3528**	−0.0219
16																**0.0468**	0.0305	0.0113	0.3552	0.0214
17																	0.0029	−0.0736	0.2148	0.0850
18																		−0.0780	0.1932	0.0884
19																			0.4219	0.0197
20																				0.4619