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ORIGINAL ARTICLE

Phylogeographic patterning in a freshwater crab species (Decapoda: Potamonautidae: *Potamonautes*) reveals the signature of historical climatic oscillations

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ABSTRACT

Aim The phylogeographic relationships among populations of the common Cape River crab, *Potamonautes perlatus*, are examined to investigate whether the contemporary population genetic structure is congruent with the hypothesized hydrographic evolution of drainage systems established during the Pliocene, or whether it reflects an older Miocene climatic amelioration.

Location 139 samples of *P. perlatus* were collected from 31 populations distributed among the five major perennial drainage systems and a number of smaller catchments in the Western and Eastern Cape, South Africa.

Methods Phylogeographic analysis using parsimony, maximum likelihood, minimum evolution and Bayesian inferences was employed for the 16S rRNA mtDNA gene region, while bootstrapping and posterior probabilities were used to assess the robustness of clades. In addition, nested clade analysis was performed in an attempt to disentangle the contemporary and historical factors that have sculpted genealogical relationships among conspecific populations of *P. perlatus*.

Results Phylogenetic topologies were congruent irrespective of the evolutionary method employed. Two highly distinct reciprocally monophyletic clades characterized by marked levels of corrected sequence divergence were present, with no shared haplotypes between the two major phylogroups. Phylogroup one comprises the populations of the westward-flowing drainages (mainly the Berg and Olifants drainages), and phylogroup two comprises all of the southward-flowing drainages and can further be divided into two subclades – one containing the Breede River populations, and the other containing the Gamtoos and Gourits drainage systems. The nested clade analysis demonstrated restricted gene flow and long-distance dispersal for a number of higher clade levels. The higher-level groups and results for the total cladogram suggest either fragmentation or isolation by distance.

Main conclusions Freshwater crabs are generally highly philopatric, and dispersal, although not common, has occurred historically. The westward-flowing drainages (Berg, Olifants, Eerste, Liesbeeck and Tokai) are isolated from the southward-flowing drainages by the Cape Fold Mountains, while the southward-flowing drainages have a number of tributaries that extend into the low-lying regions, allowing for gene flow between these three major drainages systems (Breede, Gamtoos and Gourits). Among the westward-flowing drainages, a more intensive sampling regime is required to understand evolutionary relationships. Our molecular results suggest that the observed patterns pre-date the formation of contemporary hydrographic patterns in the Cape. This suggests that an older Late Miocene event has severely impacted the contemporary population structure

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in this species, as recent Pliocene hydrographic boundaries do not correspond to the phylogeographic pattern observed. Conservation efforts for aquatic taxa should clearly be directed at the catchments, in an attempt to conserve biological diversity.

Keywords

Climate change, drainage, freshwater crabs, Miocene, nested clade analysis, palaeogeography, phylogeography, *Potamonautes perlatus*, South Africa, 16S rRNA mtDNA.

INTRODUCTION

Ubiquitous philopatric inland aquatic taxa incapable of navigating marine or terrestrial barriers and lacking active dispersal life-history stages, such as freshwater crabs (which produce a small number of lecithotrophic eggs, lack planktonic larval stages, and exhibit direct development followed by maternal care), provide an excellent opportunity to examine relationships between historical and contemporary physiography and hydrology. Freshwater crabs are generally perceived to be highly amphibious, since they are frequently observed on foraging excursions considerable distances from freshwater and are assumed to be capable of dispersing across terrestrial barriers. Banarescu (1990) argues that this renders them unsuitable for the inference of patterns of drainage evolution and demography through the examination of relationships among conspecific populations. Phylogenetic analyses of the southern African freshwater crab species (Daniels *et al.*, 2002) have, however, revealed clear biogeographical patterning, negating Banarescu's (1990) conclusion.

More recently, a phylogeographic study, using allozymes and limited 16S rRNA mtDNA sequencing, undertaken by Daniels (2003) on the widely distributed common Cape freshwater crab, *Potamonautes perlatus* revealed allozymic invariance contrasted with marked sequence divergence. This study revealed two markedly distinct clades, which comprised western and southward-flowing rivers, respectively, with the Cape Fold Mountains potentially being the barrier to gene flow. These results suggest that this species is highly philopatric and is incapable of dispersing over large geographic distances. More importantly, it appears that the contemporary genetic structure observed has been affected by historical drainage isolation and climatic changes. Limited geographic sampling (37 specimens from 10 localities) as well as the absence of samples from key drainages (such as the Gourits River) in the earlier study (Daniels, 2003) precluded the author from drawing robust conclusions with respect to intraspecific evolutionary relationships and associated processes leading to population structure among the crabs of the various drainage systems in the region. The area encompassed by the Western and Eastern Cape province has undergone large-scale Miocene climatic and environmental changes. These have included several severe marine transgressions, coupled with the development of the cold-water proto-Benguela current (Siesser, 1978, 1980), which led to considerable habitat alterations that impacted taxa, including freshwater taxa, in the region (Daniels *et al.*, 2004). The modern arrangement of drainages in the Western and Eastern Cape was established at the end of tectonic activity during the Late Pliocene (5.3 Ma) (Deacon, 1983; Partridge & Maud, 1987). The model of drainage evolution suggested by Partridge & Maud (1987) points towards a close geomorphological link between the Berg and Olifants rivers, while a close relationship is also suggested between the Breede, Gamtoos and Gourits river systems, potentially explaining faunal similarities between the drainages. It remains unclear whether the earlier Miocene climatic oscillations or the recent establishment of hydrographic boundaries during the Pliocene represent the more significant episodes influencing the evolutionary history of conspecific populations of *P. perlatus*.

In the present study, a comprehensive geographic sampling regime of *P. perlatus* populations is undertaken among the five major perennial drainages in the Cape to test whether the evolutionary relationships among allopatric populations reflect the dramatic Miocene climatic events, or whether they reflect contemporary hydrographic patterns established during the Pliocene. In the present study, we apply both phylogenetic analyses and nested clade analysis (NCA) in an attempt to understand the demographic history among conspecific populations of this taxon. NCA is a powerful statistical method that allows for a rigorous assessment of the relationship between geography and haplotype distribution and discriminates between processes that have influenced population structure (Templeton *et al.*, 1995; Templeton, 2004).

MATERIALS AND METHODS

Sample collection

The five major perennial rivers in the region are the Olifants, Berg, Breede, Gourits and Gamtoos systems. The Olifants and Berg rivers flow towards the west coast, the Breede River drains the intermountain fault basin of the Hex and Langeberg Mountains, while the Gourits River drains the Karoo Plateau and Agulhas Plain, and the Gamtoos drains the Baviaanskloof and Groot Swartberg mountains. A total of 139 *P. perlatus* samples were collected from 31 localities throughout the

Table 1 Sample localities, drainage systems, geographic positions and the number of individuals (*N*) for the *Potamonautes perlatus* populations sampled in the present study. Pop N refers to the population number as indicated on the map (Fig. 1)

Pop N	Locality	Major drainage	Coordinates		*N*
1	Citrusdal	Olifants River	32°33′23.0″ S	19°11′25.0″ E	4
2	Boontjieskloof	Olifants River	32°33′23.0″ S	19°07′50.0″ E	2
3	Kriedouwkrans	Olifants River	32°21′37.0″ S	19°00′0.0″ E	5
4	Clanwilliam	Olifants River	32°08′53.0″ S	18°56′0.0″ E	4
5	Paarl	Berg River	33°49′0.0″ S	19°03′0.8″ E	4
6	Baainskloof	Breede River	33°35′0.0″ S	19°07′0.0″ E	4
7	Tokai	–	34°00′0.0″ S	18°23′0.5″ E	3
8	Liesbeeck	–	34°11′25.0″ S	18°25′0.0″ E	5
9	Stellenbosch	Eerste River	33°45′0.0″ S	19°03′0.0″ E	5
10	Klein River	–	34°25′0.0″ S	19°32′6.5″ E	5
11	High Noon	Breede River	33°53′0.0″ S	19°04′0.0″ E	5
12	Robinson	Breede River	31°51′8.3″ S	19°52′14.6″ E	4
13	Bonnievale	Breede River	33°56′0.0″ S	20°06′0.0″ E	3
14	De Hoop	Breede River	34°23′0.0″ S	20°36′0.0″ E	4
15	Vet River	–	34°01′4.8″ S	21°13′8.9″ E	5
16	Groot River	Gourits River	33°40′15.0″ S	21°10′4.7″ E	5
17	Huis River	Gourits River	33°30′7.8″ S	21°36′2.9″ E	5
18	Dwyka River	Gourits River	33°05′1.7″ S	21°34′12.9″ E	5
19	Vlei River	Gourits River	33°33′6.6″ S	21°53′5.5″ E	4
20	Grobbelaars River	Gourits River	33°26′13.9″ S	22°14′15.5″ E	5
21	Prince Albert	Gourits River	33°07′0.0″ S	21°55′13.4″ E	5
22	Swartberg Pass	Gourits River	33°17′16.6″ S	22°02′16.2″ E	4
23	Nels River	Gourits River	33°29′10.8″ S	21°26′6.6″ E	5
24	Hankey	Gamtoos River	33°50′0.0″ S	24°52′11.7″ E	5
25	Patensie	Gamtoos River	33°35′16.3″ S	24°46′10.7″ E	5
26	Andrieskraal	Gamtoos River	33°44′15.5″ S	24°38′2.5″ E	5
27	Poortjies	Gamtoos River	33°39′10.7″ S	24°32′3.5″ E	4
28	Sand River	Gamtoos River	33°41′4.6″ S	24°35′4.9″ E	5
29	Smitskraal	Gamtoos River	33°39′12.4″ S	24°21′15.7″ E	5
30	Bosdorp	Gamtoos River	33°39′10.7″ S	24°32′3.5″ E	5
31	Kleinplaats	Gamtoos River	33°38′12.9″ S	24°27′14.2″ E	5

species range in the Western and Eastern Cape, South Africa (Table 1; Fig. 1). This includes data, from 37 specimens representing 10 populations, published in an earlier study by Daniels (2003). Between two and five animals were collected per locality, using ox-heart-baited lines. Two recent studies by Morando *et al.* (2003) and Sinclair *et al.* (2004) suggest that sample sizes as low as five or fewer animals per population are sufficient for phylogeographic studies. In addition, financial and time constraints precluded a more intensive sampling regime. We therefore attempted to sample populations that cover most of the distribution range of the species, with fewer samples per site. Crabs were killed by freezing at −20 °C overnight, at which point muscle tissue from the periopods was extracted and placed into absolute ethanol. Alternatively, periopods were broken from living crabs in the field, and the muscle tissue dissected and placed directly in 96% ethanol, after which time crabs were returned to the river system. These ethanol samples were kept on the bench top in the laboratory until required for genetic analysis.

DNA sequencing and phylogenetic analyses

A detailed description of the DNA extraction protocol employed in the present study is outlined in Daniels *et al.* (2002). Briefly, tissue samples were washed in distilled water to remove the excess ethanol. Then, a portion of the tissue was placed in a reaction vessel with DNA lysis buffer and 20 μL of proteinase k and left overnight at 55 °C. Thereafter, a standard phenol-chloroform extraction was performed, followed by precipitation of the DNA in ice-cold absolute ethanol containing 45 mM of ammonium acetate. DNA pellets were resuspended in distilled water, and a 1 in 20 dilution was used in the polymerase chain reactions (PCRs). The primers 16Sa (5′-CGC CTG TTT ACT AAA AAC AT-3′) and 16Sb (5′-CCG GTC TGA ACT CAG ATC ACG T-3′) were used to amplify the 16S rRNA gene (Cunningham *et al.*, 1992). The 16S rRNA mtDNA locus has been used extensively in phylogeographic studies on crustaceans (Daniels, 2003; Fetzner & Crandall, 2003) since the variation in the loop regions is unconstrained. For each PCR, a 25-μL reaction was performed that contained 14.9 μL of Millipore water, 3 μL of 25 mM $MgCl_2$, 2.5 μL of 10X Mg^{2+} free buffer, 0.5 μL of a 10- mM dNTP solution, and 0.5 μL of each of the primers at 10 mM, 0.1 U of *Taq* polymerase, and 1 to 3 μL of template DNA. The PCR temperature regime for the gene fragment was 95 °C for 2 min; 95 °C for 30 s; 48 °C for 40 s; 72 °C for 1 min; and then 32 cycles for the last three steps, followed by a final extension of 10 min at 72 °C. PCR products were electrophoresed in a 1% regular agarose gel containing

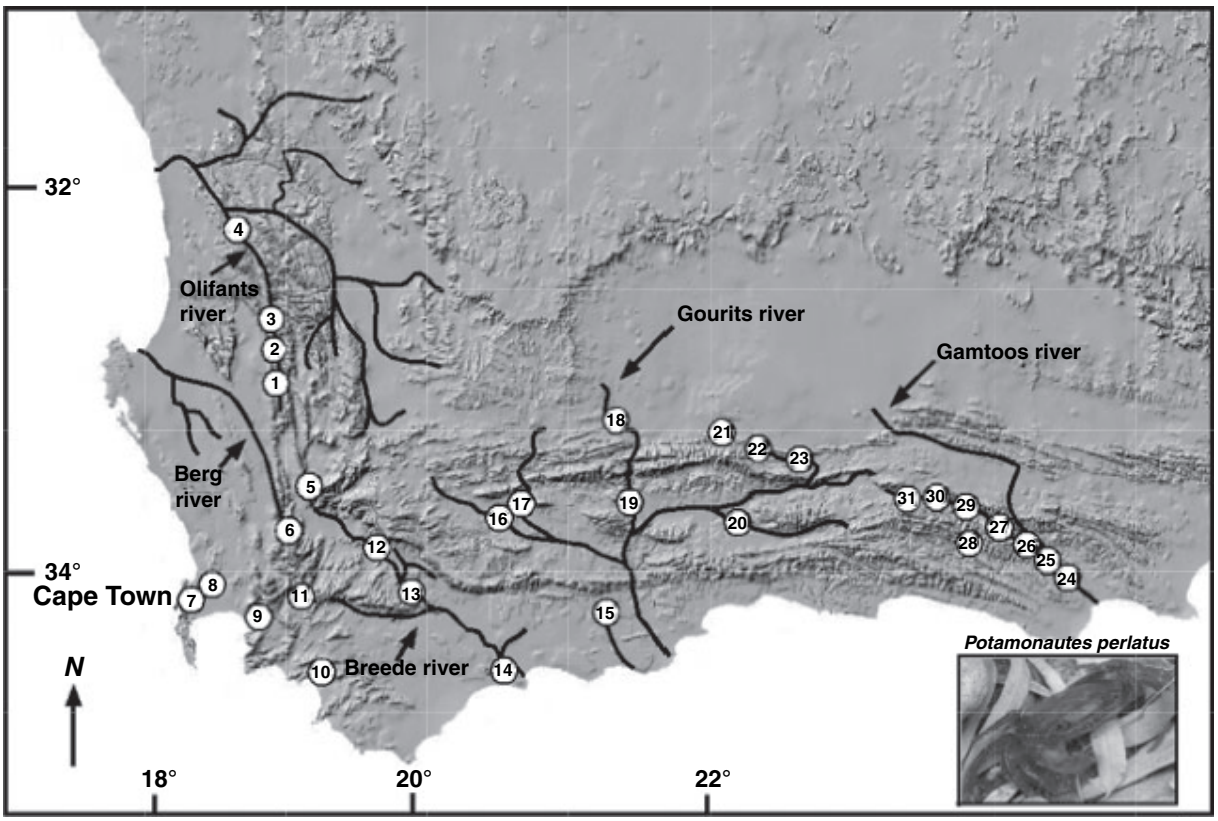

Figure 1 Map showing the distribution of the 31 *Potamonautes perlatus* (inset picture) populations sampled among the five perennial drainages throughout the Western and Eastern Cape, South Africa (1 cm = 32 km). The locality numbers correspond to those provided in Table 1.

ethidium bromide for 30 min at 70 V. Products were visualized under UV light. PCR products were purified using a PCR purification kit (QIAgen, Valencia, CA, USA). Purified PCR products were cycle-sequenced in both directions using standard protocols. Unincorporated dideoxynucleotides were removed by gel filtration using Sephadex G-25 (Sigma, St Louis, MO, USA). Sequencing was performed on an ABI 3730 automated machine.

Phylogenetic analyses

An earlier phylogenetic study (Daniels *et al.*, 2002) of the southern African Potamonautidae demonstrated that *P. perlatus* fell in a well-supported clade of large- bodied freshwater crabs that included *P. warreni* (GenBank number AY042251). Consequently, this taxon was used as an outgroup. Aligned forward and reverse sequences were checked for base ambiguity in Sequence Navigator (Applied Biosystems, Farmingham, MA, USA) and a consensus sequence was created for each sample. The 16S rRNA sequences were aligned in CLUSTAL X (Thompson *et al.*, 1997) using the default parameters of the program and were further adjusted by eye where obvious mismatches were made by the computational alignment. These sequences were combined with those from the earlier study undertaken by Daniels (2003) – accession numbers for this earlier study were AF493160–AF493176. Phylogenetic data analyses were executed in PAUP*4 version beta 10 (Swofford, 2002) using parsimony (MP) and minimum evolution (ME) optimality criteria, as well as maximum likelihood (ML). For the MP analysis, trees were generated using the heuristic search option with TBR branch swapping using 1000 random taxon additions. For the MP analysis, gaps were excluded as characters, and all character changes were weighted equally. For the ML analysis, the best-fit substitution model was calculated using MODELTEST version 3.06 (Posada & Crandall, 1998), as selected using the Akaike information criterion (AIC) (Akaike, 1973). This reduces the number of unnecessary parameters that contribute little to describing the data by penalizing more complex models (Bernham & Anderson, 2002; Nylander *et al.*, 2004; Posada & Buckley, 2004). Corrected sequence divergence values between haplotypes for the ME analysis were calculated using the ML model. The ML search was also heuristic, with 100 random sequence additions.

Phylogenetic confidence in the nodes recovered was estimated by bootstrapping (Felsenstein, 1985), analysing 1000 pseudo-replicates of data sets for MP, 10,000 bootstrap pseudo-replicates for ME, and, owing to computational constraints, only 100 pseudo-replicates for ML. Bayesian inferences were used to investigate optimal tree space using the program MRBAYES 3.0b4 (Ronquist & Huelsenbeck, 2003). For each analysis, four Markov chains were run, with each chain started from a random tree and six million generations

generated. Sampling from the chain occurred at every 5000th generation. MODELTEST was used to select the appropriate substitution model. A 50% majority rule consensus tree was generated from the trees retained after the burn-in trees were discarded, with posterior probabilities for each node estimated by the frequency at which the node was recovered. Topological congruence between all tree-building methods indicates a more accurate phylogenetic inference, and hence all these methods were employed. To test the adherence of a molecular clock for our sequence data we used a log-likelihood ratio test [using the Shimodaira–Hasegawa test: Shimodaira & Hasegawa (1999)] in PAUP* that compares likelihood scores generated under the assumption of a molecular clock with the unconstrained likelihood, i.e. without a molecular clock assumption.

Nested clade analysis

To determine the relationships among haplotypes, we used a nested clade analysis (NCA) (Templeton *et al.*, 1995; Templeton, 2004). A 95% probability haplotype cladogram was constructed according to Templeton & Sing (1992) and Crandall & Templeton (1996) using the software TCS version 1.13 (Clement *et al.*, 2001). This network was then converted into a nested statistical design as outlined in Templeton & Sing (1993) and Crandall & Templeton (1996). Geographic and

Table 2 Distribution of the 33 haplotypes across the 31sampled populations of *Potamonautes perlatus*

	Haplotypes															
Locality	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Tokai	3															
Clanwilliam		1			3											
Paarl			1	1	2											
Citrusdal					4											
Kriedouwkrans					5											
Boontjieskloof					2											
Liesbeeck						5										
Stellenbosch						2	3									
Andrieskraal								1	3							
Sandrivier									5							
Patensie									1							
Hankey									3				1			
Bosdorp									1	4						
Poortjies										4						
Vet River											2	2				
Grobbelaars River														1	1	3
Groot River															1	
Huis River																1

	Haplotypes																
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Hankey	1																
Andrieskraal		1															
Patensie		4															
Groot River		1	3														
Swartberg Pass		1	3														
Nels River		1	4														
Vlei River		1	4														
Dwyka River		1	4														
Smithskraal			1	4													
Huis River			3			1											
Klein River			1		1	3											
Prince Albert			5														
Kleinplaats				5													
De Hoop						2					1	1					
Highnoon							5										
Bainskloof								1	1	1							1
Robertson													1	1	1	1	
Bonnievale																3	

genetic associations were tested using the software GEODIS version 2.0 (Posada *et al.*, 2000), under the null hypothesis of no geographic association among haplotypes using 10,000 permutations. Two statistics were estimated: the clade distance (D_C), which measures the geographic spread of a clade, and the nested clade distance (D_N), which measures how a clade is geographically distributed relative to other clades in the same higher-level nesting category. Templeton's (2004) inference key was then used to interpret patterns of population structure.

RESULTS

We amplified a 404-base-pair fragment of the 16S rRNA mtDNA gene region. The 102 new sequences were deposited in GenBank under the accession numbers DQ028635–DQ028734 and combined with previous data from Daniels (2003) for a total data set of 139 individuals. These data resulted in a total of 33 unique haplotypes (Table 2). The parsimony analyses recovered 100 equally parsimonious trees with a tree length of 65 steps, a CI = 0.87, and an RI = 0.93. The model selected using MODELTEST was TVM + I ($-\ln L = 950.31$) (AIC = 1916.63). The base frequencies were $A = 0.3675$; $C = 0.0884$; $G = 0.1876$ and $T = 0.3565$. The rate matrix was R(a) [A-C] = 3.0950; R(b) [A-G] = R(e) [C-T] = 5.3624; R(c) [A-T] = 0.6889; R(d) [C-G] = 5.0927 and R(a) [G-T] = 1.0000. The proportion of invariable sites (I) was 0.6712, while equal rates were applied among variable sites. The ML topology was highly congruent with the topology derived from MP, ME and Bayesian inferences. Consequently, only the ML topology is shown (Fig. 2). Two statistically well-supported, highly divergent, reciprocally monophyletic phylogroups were evident. Clade 1 comprised seven haplotypes, all of which belong to westward-flowing drainages, and included samples mainly from the Berg and Olifants river systems, as well as the Tokai, Eerste (Stellenbosch) and Liesbeeck rivers. Clade 2 comprised 26 haplotypes and contained all the populations from southward-flowing drainages. Clade 2A comprised all the samples from the Gourits and Gamtoos drainages, while clade 2B comprised samples from the Breede River system. All three of these drainage systems drain the interior plateau and flow southwards. The maximum corrected

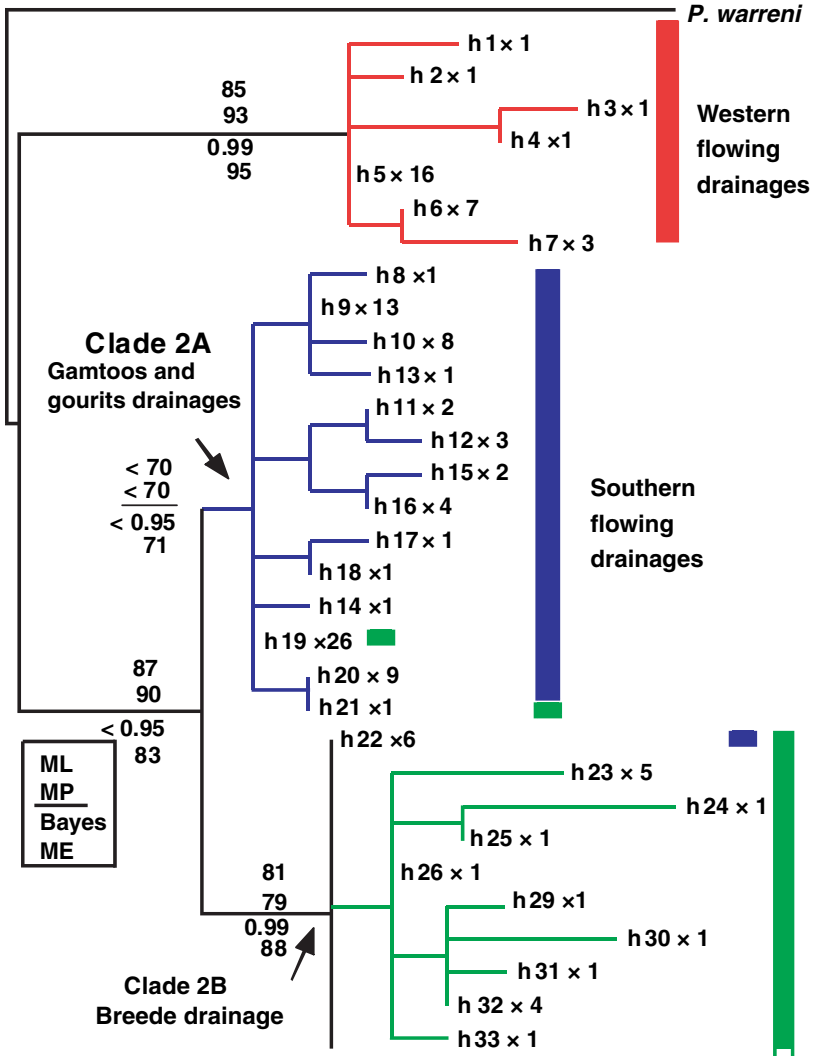

Figure 2 A maximum likelihood phylogram based on the TVM + I model ($-\ln L = 950.31$) (AIC = 1916.63) derived from the 16S rRNA mtDNA sequences for the 33 haplotypes found among the 31 populations of *Potamonautes perlatus*. Bootstrap values for maximum likelihood and maximum parsimony are given above each branch, while posterior probabilities and bootstrap values from the Bayesian analysis and minimum evolution are given below the branch. Bootstrap values < 70% and posterior probabilities < 1.00 are not shown. Two highly divergent clades are evident: these are the westward- and southward-flowing drainages. Within the southward-flowing drainages, two clades, 2A and 2B, comprise the Gamtoos and Gourits, and the Breede rivers, respectively. The numbers h1–h33 on the phylogram represents the 33 observed haplotypes, listed in Table 2. Where two colours are present (in clade 2) next to a haplotype, that haplotype occurs in both major drainages.

sequence divergences within clades 1 and 2 were 1% and 3%, respectively, while the divergence between the two clades was 6%. The likelihood ratio test demonstrated that the unconstrained tree was not statistically different from one constrained by the molecular clock assumption (947.97–948.29 = 0.31912; $P = 0.3693$), allowing the application of a molecular clock to our data to estimate divergence times. Application of a molecular clock in the present study [using 16S rRNA calibrations of 0.53%/Myr for porcelain crabs (Stillman & Reeb, 2001), or 0.96%/Myr for fiddler crabs (Strumbauer *et al.*, 1996), or 0.65%/Myr for grapsid crabs (Schubart *et al.*, 1998)] suggests a Late Miocene divergence (11.32–6.25 Ma) between the two major clades, while divergence between the two subclades in clade 2 occurred during the Early Pliocene (5.66–3.12 Ma).

Nested clade analysis

A statistical parsimony network constructed using TCS demonstrates that three main clades are evident among the haplotypes (Fig. 3). The clade distances (D_C) for clades 1-2, 1-10, 2-3, 2-4, 2-7, 3-3 and 4-2 (Fig. 4) are significantly small, reflecting an under-dispersed geographic spread of haplotypes within these clades (results not shown). The nested clade distances (D_N) show reversals from this pattern, with one or two D_N values being significantly large ($P < 0.05$). These values for clades 1-10, 1-2, 2-3 and 4-2 reflect instances in which a clade of geographically confined haplotypes is well separated from the geographic centre of the more inclusive nesting clades (Table 3). The 33 haplotypes were partitioned into 20 one-step clades, eight two-step clades, four three-step clades and two four-step clades. The haplotype cladogram revealed two highly divergent groups (clades 4-1 and 4-2) comprising the westward-flowing drainages and the southward-flowing drainages. Contingency analysis indicated no significant association between nested clades and geography ($P > 0.05$) except for two one-step clades, namely 1-2 and 1-10. For the former, inadequate geographic sampling precluded any conclusive outcome, whereas in the case of the latter clade a pattern consistent with restricted gene flow and isolation by distance was observed (Table 4). Haplotypes in this clade (1-10) are from the Gamtoos and Gourits drainage systems. Among the two-step clades, four clades had significant chi-square (χ^2) values. In the case of two of these clades (2-2 and 2-7), inadequate geographic sampling precluded any conclusive outcome. In the case of 2-3, we observe restricted gene flow and long-distance dispersal. Again, this is mainly between the Gamtoos and the Gourits rivers. For clade 3-3, inadequate geographic sampling precluded a conclusive outcome, while in the case of clade 4-2 we observe long-distance colonization with possible subsequent or past fragmentation by range expansion. The total cladogram indicates that the

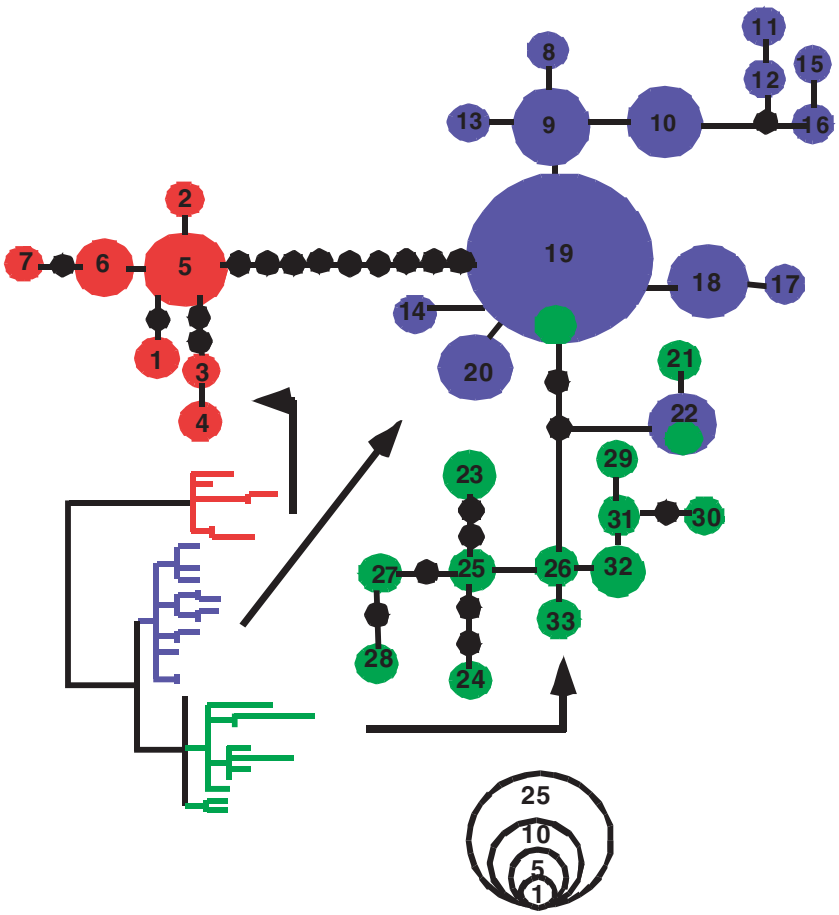

Figure 3 A statistical parsimony network showing the distribution of the 33 haplotypes. The numbers inside the circles correspond to the haplotypes listed in Table 2. The large black circles represent unsampled or missing haplotypes. The red circles represent the westward-flowing drainages (Berg and Olifants rivers), while the blue circles (Gamtoos and Gourits rivers) and green circles (Breede river) represent the southward-flowing drainages. The open circle with numbers below the parsimony network represents the number of individuals sampled for each haplotype.

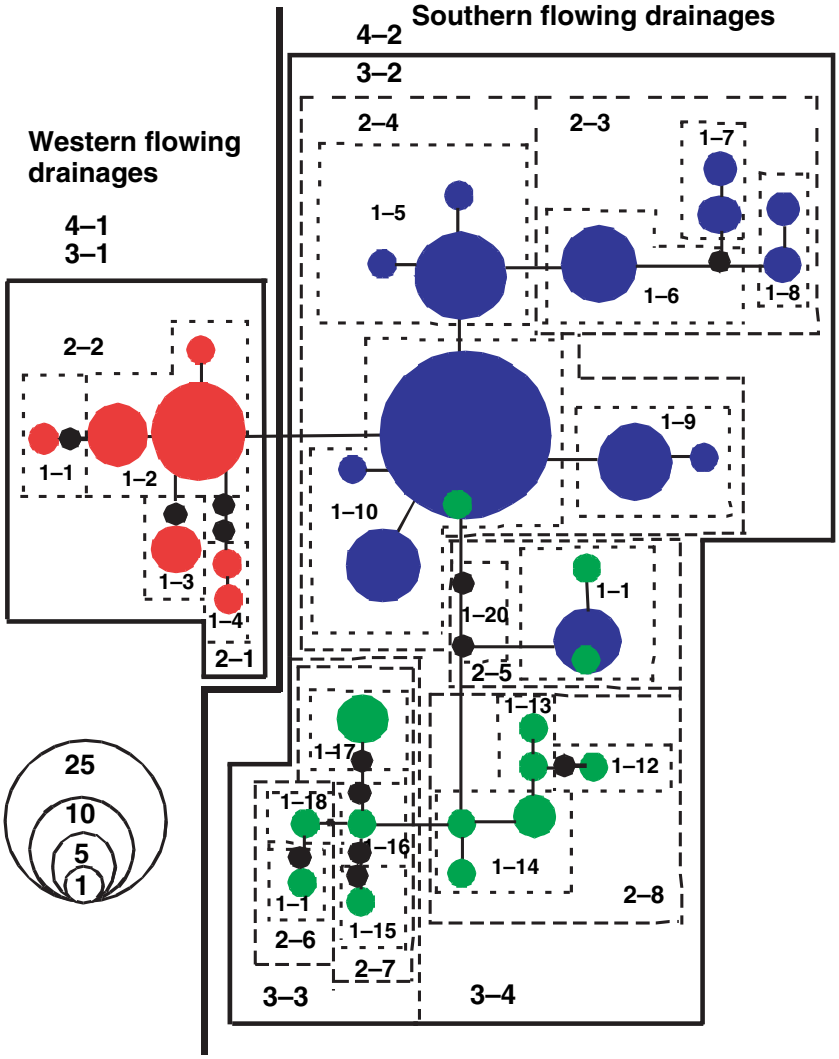

Figure 4 Nested haplotype design based on the 33 haplotypes of *Potamonautes perlatus*. The numbers inside each circle represent the haplotype number, while the size of the haplotype represents the haplotype frequency. A dark circle on a line represents a single mutation or indicates a missing intermediate. The level of nesting is 1-*x* for one-step clades, 2-*x* for two-step clades, 3-*x* for three-step clades and 4-*x* for four-step clades. The dark line between the two major groups (westward- and southward-flowing drainages) denotes a 10 mutational step difference between the two major clades. The red circles represent the westward-flowing drainages (Berg and Olifants rivers), while the blue circles (Gamtoos and Gourits rivers) and green circles (Breede river) represent the southward-flowing drainages. The open circle with numbers below the parsimony network represents the number of individuals sampled for each haplotype.

current sampling design is inadequate to distinguish between past fragmentation and isolation by distance (Table 4).

DISCUSSION

Both the phylogenetic and NCA patterns derived from the 16S rRNA mtDNA data demonstrated remarkable congruence with respect to levels of population subdivision and suggest regional philopatry between conspecific populations of *P. perlatus*. Two highly discrete, reciprocally monophyletic lineages were recovered with high statistical support (both bootstrap and posterior probabilities), and were characterized by marked sequence divergence (Fig. 2), with no shared haplotypes between the two major clades. These two major groupings coincide well with the topography of the area, since the drainages are separated by the Cape Fold Mountains.

The phylogenetic results clearly demonstrate important historical connections and dissociations among the populations of this freshwater crab. For example, the westward-flowing drainages share the same mountain catchment regions, while the southward-flowing drainages drain the interior plateau and are separated into at least two intermountain basins. Few published studies on aquatic taxa are available from this geographic area for comparison. Nevertheless, our results are broadly congruent with work undertaken on the population genetics of the Cape galaxid fish (*Galaxia zebratus*), which suggests similar levels of historical isolation between the westward-flowing and southward-flowing drainages (Waters & Cambray, 1997). In addition, unpublished results for the freshwater fish *Sandelia capensis* suggest a similar pattern of genetic differentiation (M. Cunningham and E. Swartz, pers. comm.).

Our divergence estimates date the split between the two major clades as having occurred during the Miocene. The Miocene epoch in South Africa is characterized by major climate changes. The development of the cold-water, proto-Benguela upwelling current along the west coast of southern Africa during the Late Miocene (6 Ma) (Siesser, 1978, 1980) was probably instrumental in the divergence of the two observed phylogroups. These results are not unexpected, since the Cape experienced several severe marine transgressions and regressions throughout the Miocene. It is reported that these

Table 3 Results of the NCA of *Potamonautes perlatus* 16S rRNA mtDNA haplotypes based on 10000 permutations. Clade (D_C) and nested clade (D_N) distances are given. S indicates that the distance is significantly small at the 5% level and L indicates that the distance is significantly large. In clades with both tip (T) and interior (I) nested clades, the average I-T is given. Shaded regions indicate significantly large or small D_C and D_N values

0 step clades			1 step clades			2 step clades			3 step clades			4 step clades		
Haplotypes	D_C	D_N	Clade	D_C	D_N	Clade	D_C	D_N	Clade	D_C	D_N	Clade	D_C	D_N
7			1-1	0.0^S	70.4^S	2-2	90.1	90.4	3-1			4-1	89.3^S	246.0^L
6	26.7^S	133.5^L	1-2	80.6^S	89.3									
2	0.0	85.7												
5	35.4^S	62.9^S												
I-T	32.8^S	−1.3												
1			1-3	0.0^S	106.4									
			I-T	80.6^L	1.0									
3			1-4			2-1	0.0	73.2						
4														
						I-T	90.1	17.2						
10			1-6	0.0^S	148.3	2-3	135.7	136.5	3-2	60.0^S	257.9^L	4-2	165.9^S	171.9^S
11			1-7	0.0^S	162.0^L									
12														
15	51.2	51.2	1-8	36.0^S	94.8^S									
16	22.3	28.4												
I-T	−28.9	−22.8												
			I-T	−19.6	22.9									
13	0.0	20.8	1-5	12.5^S	152.5	2-4	140.1	140.1						
9	11.9	12.4												
8	0.0	4.6												
I-T	11.9	−0.2												
17	0.0	163.3	1.9	143.1	142.7									
18	139.5	141.3												
I-T	139.5	−21.9												
14	0.0	12.1^S	1-10	106.3^S	134.2									
19	48.5^S	82.0^S												
20	4.6^S	190.4^L												
I-T	−42.0	111.0^L												
			I-T	−103.0^S	16.2	I-T	4.4	3.6						
21	0.0	59.7	1-11			2-5	66.7	116.7	3-4	139.3^S	139.3^S			
22	67.8	67.8												
I-T	67.8	8.1												
30			1-12	0.0	170.5	2-8	111.7	108.8						
29			1-13	0.0	55.4									
31														
26	0.0	98.1	1-14	74.7	82.4									
32	98.9	116.1												
33	0.0	98.1												
I-T	−79.1	−14.4												
			I-T	74.7	−11.3	I-T	−45.0	7.9						
24			1-15	0.0	146.2	2-7	15.0^S	41.4^S	3-3	111.8^S	232.7^L			
25			1-16	0.0	146.2									
23			1-17	0.0^S	58.6^S									
			I-T	0.0^L	73.1									
27			1-18			2-6	0.0	115.9						
28			1-19											
						I-T	15.0	−74.5^S						
									I-T	−74.8^S	103.3^L			
												I-T	76.6^L	−74.1^S

Table 4 Results from the nested clade analyses for the 31 populations of *Potamonautes perlatus* collected from the five perennial drainages in the Western and Eastern Cape, South Africa. Nested contingency results are based on 10,000 permutations in GEODIS (Posada *et al.*, 2000). An asterisk indicates significance at the $P < 0.05$ level. Inferences were made using Templeton's (2004) key

Clade	χ^2	Probability	Inference chain	Inferred pattern
1-2	28.87	0.00*	1-19-20-No	Inadequate geographic sampling
1-10	67.69	0.00*	1-19-20-2-11-17-4-No	RGF/IBD
2-2	46.50	0.00*	1-19-20-No	Inadequate geographic sampling
2-3	38.00	0.00*	1-19-20-2-3-5-6-7-Yes	RGF with some LDD
2-4	74.00	0.00*	1-19-20-2-11-12-No	Range expansion
2-7	7.00	0.03*	1-19-20-No	Inadequate geographic sampling
3-3	9.00	0.06	1-19-20-No	Inadequate geographic sampling
4-2	164.00	0.00*	1-19-2-3-5-6-13-Yes	LDC with possible subsequent or past fragmentation by range expansion
Total	139.00	0.00*	1-19-20-2-3-5-15-16-18-Yes	Geographic sampling inadequate to discriminate between fragmentation and IBD

RGF, restricted gene flow; IBD, isolation by distance; LDD, long-distance dispersal; LDC, long-distance colonization.

drops and subsequent increases in sea level were in the region of +200 m during the Early Miocene, while during the Late Miocene levels dropped 150 m and then a further 100 m compared with the current level during the Pliocene (Dingle & Hendey, 1984). These events, coinciding with dramatic climate changes, significantly affected the population history of both terrestrial and inland aquatic taxa inhabiting the region. For example, while the Miocene was characterized by high levels of precipitation, the Pleiocene/Pleistocene epochs were characterized by markedly arid conditions. Reduced precipitation is a factor that is likely to have limited the dispersal capacity of freshwater taxa, including freshwater crabs. It is likely that periods of high rainfall facilitated widespread dispersal of freshwater crabs, since these animals are capable of short-distance terrestrial dispersal, while periods of low rainfall would have resulted in the opposite condition. Casual observations (S. Daniels pers. obs.) suggest that terrestrial dispersal for freshwater crabs is possible under conditions of high humidity. Furthermore, many headwater subpopulations are connected through the shared mountain catchments they drain. For example, both the Berg and Olifants rivers drain proximate high-altitude areas of the Cederberg Mountains – with both these drainages forming part of the clade comprising the westward-flowing drainages – while the Gamtoos, Gourits and Breede rivers drain the interior plateau of the mountains and form the well-supported clade 2. Nevertheless, a cautionary approach should be undertaken when using molecular clock calibrations (Graur & Martin, 2004). More importantly, congruent patterns of genetic cladogenesis present within unrelated faunistic groups sampled from the same geographic region, such as freshwater isopods (*Mesamphisopus*) and cordylid lizards (*Cordylus*), all support a Miocene divergence between taxa (Daniels *et al.*, 2004; Gouws *et al.*, 2004). Linder (2003) reported similar Late Miocene divergences and radiation among southern African floral genera such as *Phylica* and *Ehrharta*. It is interesting that the major Miocene divergence between the two observed clades contradicts the more recent Pliocene drainage isolation proposed by Partridge & Maud (1987), suggesting that this later historical event has been restricted to evolutionary differentiation among conspecific populations within each of the two major clades.

Nested clade analysis revealed significant geographic structure at several levels (Fig. 3; Table 4). These results suggest that we can clearly reject the null hypothesis of no geographic association for the observed genetic results in *P. perlatus*, since the species has a low proclivity for dispersal.

The question that now arises is why there is no large-scale gene exchange between the two major clades evident in our study. The most plausible explanation is the difference in elevation between the two regions, or, alternatively, these results may reflect historical isolation between the drainage systems. The monophyly of these two major clades, and the general level of sequence divergence within each clade suggest relatively long periods of historical isolation. Allozyme electrophoresis data from the Berg, Breede, Olifants and Gamtoos (excluding the Gourits River) populations suggest genetic invariance across the distribution range of *P. perlatus*, strongly contrasting with the results from the sequencing analysis (Daniels, 2003). This indicates that these markers are probably too conservative to detect the population divergences observed with mtDNA sequence data, or, alternatively, it suggests that there may be sex-biased dispersal in this group. In addition, selection at the allozyme level may be responsible for maintaining allele frequency homogeneity among the allopatric populations.

Nuclear markers, in the form of micro-satellites or amplified fragment length polymorphisms, in combination with extensive fine-scale geographic sampling, may illuminate historical relationships within and between these two major clades. It has been demonstrated that inferences about gene flow, or the lack thereof, are heavily dependent on the sample sizes and assumptions of coalescent theory (Morando *et al.*, 2003). While it has been suggested that the sampling of at least five animals per population would be adequate to detect gene flow in taxa with low dispersal capacity, Sinclair *et al.* (2004) suggest that even fewer samples are needed to detect deeper coalescent events. Large-scale flooding plumes over short temporal scales may

promote gene exchange between drainages, particularly among the lower-lying areas of the Gamtoos and the Gourits drainages. There is great aquatic faunistic similarity, particularly of the ichtyofauna, between the Breede and Gourits rivers, which is thought to reflect various tributary captures. This may also be indicative of a recent shared hydrograhic connection. Geographically, the upper tributaries of the Berg River are close to those of the Breede River, but we found no evidence of gene flow between the rivers. Our ability to detect such gene flow is, however, limited by the small sample sizes from the Berg River. One of the major tributaries of the Olifants River, the Doring River, bisects the Little Karoo and is in certain places geographically close to the tributaries of the Gourits River, such as the Touws River. However, we observed no haplotypes that would potentially link these drainages.

Conservation priority for these aquatic ecoregions in the Western Cape is of particular importance since the region harbours a large number of endemic endangered freshwater fish species and is generally considered to be a diversity hotspot. Using the results from the present study as a template, it appears that the river system as a whole, including all its drainage basins and tributaries, should be considered worthy of conservation, particularly since widely distributed taxa may contain high levels of genetic differentiation (Davies *et al.*, 1993). In this respect, attempts at inter-drainage water transfers should be limited or stopped since they may artificially enhance genetic exchange between previously isolated populations. While the transfer of inland aquatic organisms for use as bait during angling has not been demonstrated in South Africa, this practice should be discouraged, as it may also artificially aid dispersal of previously isolated groups of organisms and may have negative genetic and ecological consequences. Clearly, large-scale phylogeographic studies are required to identify isolated taxa among these distinct drainages in order to understand their unique evolutionary histories and to develop robust conservation plans. This is particularly important for taxa occurring in low-lying regions and that exhibit low vagility, including taxa that are classed as endangered or vulnerable to extinction.

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