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Multilocus coalescent species delimitation reveals widespread cryptic differentiation among Drakensberg mountain-living freshwater crabs (Decapoda : *Potamonautes*)

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Abstract. Cryptic lineages present major challenges for evolutionary and conservation studies, particularly where these lineages remain undiscovered. Freshwater crabs are known to harbour cryptic diversity, in most cases with limited morphological differences. During the present study, we used a multilocus (12S rRNA, 16S rRNA, COI, 28S rRNA, DecapANT and PEPCK) Bayesian species delimitation to examine cryptic diversity within a freshwater crab species complex (*Potamonautes clarus*/*P. depressus*). We sampled 25 highland rivers in the Tugela and uMkomazi River drainage systems of the Drakensberg Mountain range, in the KwaZulu–Natal province of South Africa. Our results showed there to be at least eight lineages: six novel potamonautid freshwater crabs, and two described taxa *P. clarus* and *P. depressus*. Divergence from the most recent common ancestor occurred between the mid- and late Miocene (12.1 Mya), while divergence within the species complex occurred ~10.3 Mya up until the Holocene (0.11 Mya). The discovery of six novel lineages of freshwater crabs from a seemingly restricted distribution range has conservation implications, but to date most conservation planning strategies have focussed on freshwater vertebrates. By conducting a fine-scale phylogenetic survey using invertebrates, this study provides a platform for the inclusion of freshwater invertebrates in future conservation assessments.

Additional keywords: Bayesian phylogenetics and phylogeography, conservation, cryptic species, divergence time estimation.

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Introduction

Accurately delineating species boundaries is central in evolutionary and ecological studies, especially those studies that involve the assessment of biodiversity and the recognition of areas of conservation priority (Sites and Marshall 2003, 2004; Agapow *et al*. 2004; Witt *et al*. 2006; Bickford *et al*. 2007; Carstens and Dewey 2010; Camargo *et al*. 2012; Camargo and Sites 2013). What constitutes a species has been given a great deal of attention in literature (de Queiroz, 1998, 2007; Hey 2001; Bauer *et al*. 2011; Fujita and Leaché 2011; Camargo *et al*. 2012). However, it is generally accepted that species can be defined as separately evolving metapopulations that are rendered monophyletic, are reproductively isolated, and morphologically distinct (de Queiroz, 1998, 2007). One major obstacle in species delimitation studies is the presence of cryptic lineages. These lineages are difficult to morphologically identify despite the evolutionary processes that have occurred during the speciation process within the species complex (Colborn *et al*. 2001; Bickford *et al*. 2007; Puckridge *et al*. 2013). Consequently, identifying species exclusively on the basis of morphological characters can lead to erroneous taxonomic classifications because subtle interspecific morphological differences can easily be overlooked (Colborn *et al*. 2001). It has therefore become imperative to evaluate operational taxonomic units, not only based on morphological characters, but also on molecular data to uncover the true evolutionary histories of speciation among cryptic lineages (Bickford *et al*. 2007; Padial and de la Riva 2009).

While molecular data are informative with regards to the hierarchical relatedness and the relative rates of evolution between species, they often provide poorly resolved phylogenetic trees because of the presence of recently diverged species (Brito and Edwards 2009; Yang and Rannala 2010). This is because the conventional use of genetic information for species delimitation relies on genetic distances (advocated by the barcoding initiative) or the monophyly of gene trees (Sites and Marshall 2003, 2004; Camargo and Sites 2013). Moreover, traditional methods of species delimitation consist of analysing multiple loci in concatenated datasets, forming a supermatrix, under the assumption that the different loci share the same species tree topology (Kubatko and Degnan 2008; Degnan and Rosenberg 2009; Heled and Drummond 2010). However, it has been demonstrated that species tree inference using concatenated datasets had some limitations (Kubatko and Degnan 2008; Degnan and Rosenberg 2009) and that it involves the subjective separation of species boundaries

(Hey 2009) without statistical exploration (Knowles 2009; Leaché and Fujita 2010; Camargo and Sites 2013; Rannala and Yang 2013). Hence, coalescence-based species tree inference using independent multiloci and Bayesian analyses have become widely accepted as robust methods for delineating species (Rannala and Yang 2003, 2013; Liu and Pearl 2007; Liu 2008; Edwards 2009; Kubatko *et al*. 2009; Liu *et al*. 2009; Heled and Drummond 2010; Knowles and Kubatko 2010; O'Meara 2010; Yang and Rannala 2010; Ence and Carstens 2011; Camargo and Sites 2013). The coalescent in the Bayesian approach acts as a prior distribution for the gene tree (Heled and Drummond 2010). Estimating species trees (barriers for gene flow) with this approach has been reported to outperform data concatenation-based species tree inferences (Kubatko and Degnan 2008; Liu 2008; Kubatko *et al*. 2009; Heled and Drummond 2010). One of the reasons for this is that the coalescent species tree model autonomously infers the genealogy of each locus and assumes that any incongruences between gene trees results from incomplete lineage sorting and that each delimited cryptic species is panmictic (Carstens and Dewey 2010; Heled and Drummond 2010; Camargo and Sites 2013; Rannala and Yang 2013). Therefore, to increase the likelihood of detecting recent lineage separation and to obtain robust evidence of lineage sorting among cryptic species in conjunction with morphology it is also essential to evaluate multiple characteristics, including multiple loci and/or genomes (Heled and Drummond 2010). Accurately delimiting species boundaries of the lineages that comprise cryptic species has implications for conservation because some already endangered species may be harbouring multiple rare and/or endemic species, which may necessitate different conservation strategies (Schönrogge *et al*. 2002; Bickford *et al*. 2007).

Cryptic species are more prevalent than previously thought and have been detected from a wide range of taxa from several different habitats (Pfenninger *et al*. 2003; Hebert *et al*. 2004; Fouquet *et al*. 2007; Murray *et al*. 2008; Lohman *et al*. 2010; Funk *et al*. 2012; Knee *et al*. 2012; Paupério *et al*. 2012; Pedraza-Lara *et al*. 2012; Cavers *et al*. 2013; Lemme *et al*. 2013; McFadden and van Ofwegen 2013; Smith *et al*. 2013; Wielstra *et al*. 2013). Nevertheless, identifying cryptic species tends to be more difficult when trying to delineate species within invertebrate taxa because, at the genus level, some tend to display low interspecific morphological divergence (Knowlton 1986, 1993; Hogg *et al*. 1998; Daniels *et al*. 2003; Pfenninger *et al*. 2003; Gouws *et al*. 2004; Witt *et al*. 2006; Seidel *et al*. 2009). This is particularly true for freshwater taxa, including decapods (Daniels *et al*. 2003; Shih *et al*. 2009; Jesse *et al*. 2010; Lemme *et al*. 2013; Phiri and Daniels 2014).

Freshwater crabs (Decapoda : Brachyura : Potamoidae) are morphologically conserved with respect to key taxonomic characters, and this often limits the delimitation of species boundaries especially in cryptic species (Daniels *et al*. 2006*a*). There is persuasive evidence suggesting that several widespread species within the sub-Saharan Africa genus *Potamonautes* (Potamonautidae) display strong morphological similarities (Barnard 1950; Daniels *et al*. 1998, 1999, 2001, 2003, 2006*b*). As such, the systematics of the genus is obscured (Daniels *et al*. 2003, 2006*b*), mostly due to the paucity of studies that also consider the use of informative genetic markers to infer the evolutionary history within the genus. In a study conducted on the species complex of two *Potamonautes* species (*P. clarus* and *P. depressus*) it was found that there were at least five distinct lineages (three undescribed) among populations of these two widespread species (Daniels *et al*. 2003). Conversely, it was previously concluded that there were only two species (Gouws *et al*. 2000), however, these conclusions (Gouws *et al*. 2000; Daniels *et al*. 2003) were based on allozyme data and a single mitochondrial (mt) DNA (16S rRNA) marker. The use of allozyme data in phylogenetic studies is now outdated and its use in phylogeographic studies are limited because similarities between alleles in allozyme data are often indeterminate, and those similarities or differences may frequently be influenced by environmental conditions. As such, while the history of population connectivity can be established, the evolutionary history of the alleles cannot be accurately assessed. The use of a single locus, mtDNA, in species delimitation studies has also been criticised. It has been argued that mtDNA alone may not reflect the true evolutionary history of a species complex, especially because of the differences in male and female mediated gene flow where only the matrilineal evolutionary history is considered (Bickford *et al*. 2007; Zink and Barrowclough 2008; Edwards and Bensch 2009) and that it does not take random lineage sorting into account (Heled and Drummond 2010). Moreover, it has also been demonstrated that mtDNA on its own, without the addition of nuclear (nu) DNA loci, can be misleading in freshwater crab phylogenetic studies (Barber *et al*. 2012). This leaves the question as to whether the five lineages (Daniels *et al*. 2003) are a true reflection of the species complex, which has prompted the re-examination of the *P. clarus*/*P. depressus* species complex.

In this study we employ Bayesian coalescence-based multilocus (mt- and nuDNA) species delimitation methods to identify species boundaries and elucidate the evolutionary history of the *P. clarus*/*P. depressus* species complex across its known distributional range in the Drakensberg Mountains in KwaZulu–Natal Province, South Africa (Fig. 1, Table 1). Moreover, we use divergence time estimations to establish the geological time scale period associated with the divergence/speciation of the lineages within the *P. clarus*/*P. depressus* species complex. The presence of defined cryptic lineages may necessitate different conservation strategies for the river catchments of the Drakensberg Mountains.

Materials and methods

Sample collection

Freshwater crab specimens were collected from 25 localities (Fig. 1, Table 1) in the Drakensberg Mountain range (KwaZulu–Natal Province, South Africa) in August and September 2011. We sampled the Tugela River ($n = 10$) and the uMkomazi River ($n = 15$) drainage systems. According to an earlier study, that sampled nine localities, *P. depressus* was found in two localities, *P. clarus* in three localities in the northern parts of the Tugela River drainage system, while the intermediate/cryptic lineages were found in four localities in the central parts of the same drainage system (Daniels *et al*. 2003). However, it is important to note that only one locality was sampled in the southern-most regions of the Tugela River

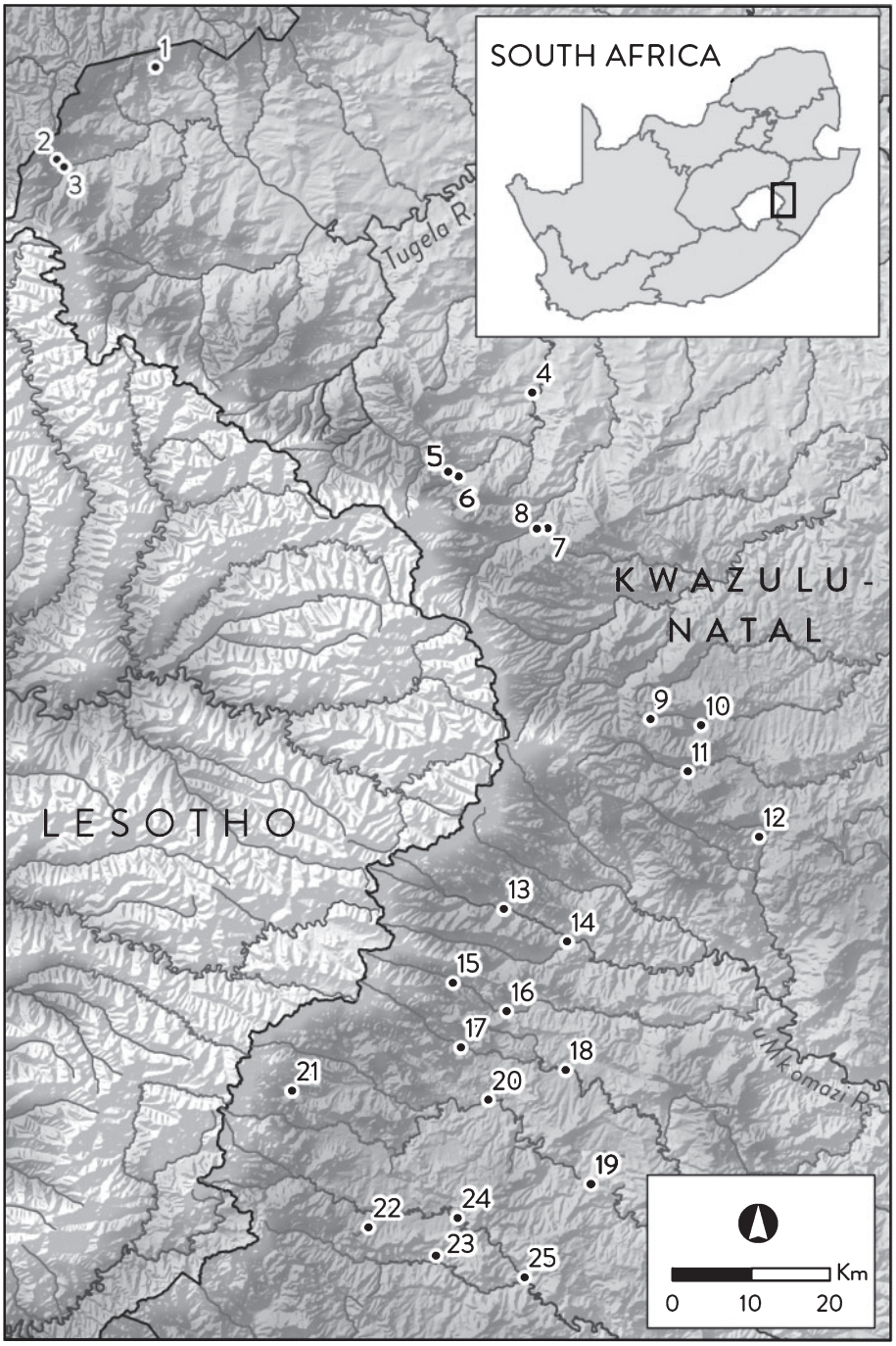

Fig. 1. Map showing the 25 localities where specimens of the *Potamonautes clarus*/*P. depressus* species complex were collected. Locality: 1, Oliviershoek Pass; 2, Gudu Falls; 3, Mahai; 4, Cathedral Peak; 5, Monk's Cowl; 6, Monk's Cowl A; 7, Injisuthi B; 8, Injisuthi A; 9, Highmoore Nature Reserve; 10, Highmoore Farm; 11, Kamberg; 12, Lotheni; 13, Vergelegen Nature Reserve; 14, Vergelegen; 15, Sani Pass; 16, Sani Pass Hotel; 17, Cobham; 18, Himeville; 19, Lower Mzimkhulu; 20, Garden Castle Nature Reserve; 21, Lower Garden Castle; 22, Bushman's Nek; 23, Ndawana; 24, Rougham; and 25, Coleford. The number of samples per site and the geographic coordinates correspond to those in Table 1. The abbreviation NR denotes nature reserves.

drainage system for *P. depressus* (Daniels *et al*. 2003), leaving a big sampling gap between the intermediates and the 'true' *P. depressus*. Moreover, the second locality where *P. depressus* was sampled is in the uMkomazi River drainage system, and no other localities/rivers were sampled within this drainage (see Fig. 1 in Daniels *et al*. 2003). Similarly, Gouws *et al*. (2000) only sampled five localities (four in the Tugela River drainage and one in the uMkomazi River drainage), and left a wide sampling

Table 1. Sampling localities (including geographical coordinates) for the *Potamonautes clarus/P. depressus* species complex and the drainage systems where sampling was conducted
N = sample size per locality

Drainage system	Collection locality (numbers are locality positions on map, Fig. 1)	*N*	Latitude (S)	Longitude (E)
Tugela River				
	1 Oliviershoek Pass	2	–28.575833	29.053667
	2 Gudu Falls	2	–28.681278	28.941333
	3 Mahai	2	–28.689611	28.949389
	4 Cathedral Peak	2	–28.947944	29.483028
	5 Monk's Cowl	2	–29.044028	29.398944
	6 Monk's Cowl a	2	–29.038694	29.387556
	7 Injisuthi b	2	–29.103361	29.501139
	8 Injisuthi a	2	–29.103444	29.488389
	9 Highmoore Nature Reserve	2	–29.321028	29.618111
	10 Highmoore Farm	2	–29.328250	29.675806
uMkomazi River				
	11 Kamberg	2	–29.381083	29.660556
	12 Lotheni	2	–29.455556	29.742250
	13 Vergelegen Nature Reserve	2	–29.538111	29.451111
	14 Vergelegen	2	–29.575333	29.523028
	15 Sani Pass	2	–29.622583	29.392806
	16 Sani Pass Hotel	2	–29.655167	29.454250
	17 Cobham	2	–29.696194	29.401972
	18 Himeville	2	–29.722250	29.521222
	19 Lower Mzimkhulu	2	–29.030361	29.831222
	20 Garden Castle Nature Reserve	2	–29.756250	29.433111
	21 Lower Garden Castle	2	–29.745722	29.209361
	22 Bushman's Nek	2	–29.901917	29.296611
	23 Ndawana	2	–29.934472	29.373611
	24 Rougham	2	–29.891167	29.398250
	25 Coleford	2	–29.958861	29.474333
	Total	50		

gap between the sampling localities within the uMkomazi River drainage system.

Crabs were captured using ox-heart baited lines and killed by freezing them for 24 h before DNA extraction. One pereopod (walking leg) was broken off from each specimen and preserved in 95% ethanol.

DNA extraction, PCR and sequencing

DNA was extracted from the muscle tissue of each pereopod using the NucleoSpin® Tissue extraction kit (Machery-Nagel, Germany) following the manufacturers' protocol. Template DNA was stored at -20°C until required for polymerase chain reaction (PCR). The DNA was diluted 1 µL : 19 µL with millipore water. We amplified three mitochondrial partial gene fragments (12S rRNA, 16S rRNA and COI) and three nuclear DNA markers (28S rRNA, DecapANT and PEPCK) – see Table S1 (in Supplementary Material) for the list of primer pairs and the PCR profiles for each of the markers. All of these markers have been extensively used in phylogenetic studies at various taxonomic levels for crustacean, including freshwater decapods (Daniels *et al*. 2002, 2006*a*, 2006*b*; Tsang *et al*. 2008; Jesse *et al*. 2010; Daniels 2011; Barber *et al*. 2012). Two specimens per locality were amplified for all six loci.

The PCR products were electrophoresed for four hours in a 1% ethidium bromide-containing agarose gel. The DNA fragments were purified using the BioFlux purification kit (Bioer Technology Co., Ltd), after which they were sent for sequencing at Macrogen Europe (The Netherlands, http://europe.macrogen.com). Sequences were checked for ambiguities and aligned with MUSCLE as executed in MEGA5 V. 2.2 (Tamura *et al*. 2011). The two coding gene fragments (COI and PEPCK) were translated to amino acids and no stop codons were detected, indicating that all sequences were valid (EMBOSS-Transeq, http://www.ebi.ac.uk/emboss/transeq/).

Sequence diversity and population relationships

The basic genetic statistics (i.e. number of haplotypes (h), haplotype diversity (*Hd*), number of variable (polymorphic) and parsimony informative sites were calculated in DnaSP V. 5.10 (Librado and Rozas 2009).

Phylogenetic reconstructions

We first reconstructed the phylogeny (gene trees) of the *P. clarus*/*P. depressus* species complex of the mtDNA (12S rRNA, 16S rRNA and COI) and the nuclear gene tree (28S rRNA, DecapANT and PEPCK) followed by a combined analyses of the mtDNA plus nuDNA datasets. For comparison,

phylogenetic trees were also constructed for the individual loci, independent of each other. Phylogenetic trees were obtained using two approaches: Bayesian inference (BI) and maximum likelihood (ML). Trees were rooted with two Western Cape mountain-living freshwater crab species (*P. brincki* and *P. parvicopus*) that are sister to the *P. clarus*/*P. depressus* species complex (Daniels *et al*. 2002). Bayesian inference (BI) were conducted in MrBayes V. 3.2. 1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003; Ronquist *et al*. 2012). Consensus BI trees were constructed for all datasets, i.e. concatenated mtDNA, concatenated nuDNA and combined concatenated mtDNA and concatenated nuDNA, in a partitioned analysis using best-fit substitution models obtained for each gene separately from jModelTest V. 2.1.3 (Posada 2008) (see Table S2 for model information). Analyses were conducted with four Markov Chain Monte Carlo (MCMC) simulations that were run for 5×10^6 generations, with each chain starting from a random tree and parameters sampled every 5×10^3 generations. Convergence was reached when the split frequency remained below 0.01 and effective sample size (ESS) values were above 100. The first 25% of the trees were discarded as burn-in and consensus trees were viewed and edited in FigTree V. 1.4 (Drummond and Rambaut 2007). Only branches with a posterior probability (p*P*) support $\geq$0.95 were considered statistically supported.

MEGA5 V. 2.2 (Nei and Kumar 2000; Tamura *et al*. 2011) was used to construct ML bootstrap trees, inferred from 5×10^3 replicates that were taken to represent the evolutionary history of the *P. clarus*/*P. depressus* species complex. We obtained nucleotide substitution models, selected by maximum likelihood, for the respective entire concatenated datasets (concatenated mtDNA, concatenated nuDNA, and the combined concatenated mtDNA and concatenated nuDNA) using MEGA5 V. 2.2 (see Table S2 for model information). Positions containing gaps and missing data were eliminated from analyses. Only branches with bootstrap support $\geq$75% were regarded as statistically well supported.

Species tree, species delimitation and divergence time estimation

The species tree topology was deduced from the multiple gene tree of the multilocus dataset using the multispecies coalescent species tree reconstruction method in *BEAST (as implemented in BEAST V. 2.0.2) (Drummond and Rambaut 2007; Heled and Drummond 2010; Drummond *et al*. 2012*a*, 2012*b*). StarBEAST uses the Bayesian Markov Chain Monte Carlo (MCMC) to estimate one species tree from individual loci by estimating the posterior of species trees under the multispecies coalescent (Heled and Drummond 2010; Drummond *et al*. 2012*a*, 2012*b*). This method attributes incomplete lineage sorting, and not gene flow, as the main cause of incongruence among multiple gene trees (Heled and Drummond 2010; Drummond *et al*. 2012*a*, 2012*b*). Compared with other species delimitation methods, *BEAST has been demonstrated to be even more accurate when used on fast evolving lineages or shallow phylogenies (Drummond and Rambaut 2007; Heled and Drummond 2010; McCormack *et al*. 2011). However, to construct the topology of the species tree, *BEAST requires that species tree is given *a priori*. We therefore used the results from the phylogenetic reconstructions (BI) of the mtDNA (Yang and Rannala 2010) to define groups to be mapped as species. From the phylogenetic reconstructions, we identified eight well supported clades. From these identified clades we only used two sequences per locality within nodes that were supported by a posterior probability of 1.00 to represent the sampled genetic diversity from the representative species. All loci (12S rRNA, 16S rRNA, 28S rRNA, COI, DecapANT, PEPCK) were included in the analysis as six independent partitions. The XML input file was created in BEAUti V. 2.0.2 (included with the BEAST software package), with each gene as a separate partition. Ploidy differences between the mt- and nuDNA genomes were assigned in BEAUti to account for the smaller effective population size of the mtDNA. The Yule speciation process was used to estimate the species tree. Four MCMC chains were run for 5×10^7 iterations, with chains and trees sampled every 5×10^6 generations. The analysis was repeated four times to confirm consistency between runs. Convergence was checked in Tracer V. 1.5, where ESS $\geq$200 for the combined runs was considered as sufficient posterior sampling (Drummond and Rambaut 2007; Drummond *et al*. 2009). Tree files were combined in LogCombiner V. 2.0.2 and maximum clade credibility trees were generated in TreeAnnotator V. 2.0.2 with mean heights, after discarding 10% of the trees as burn-in. Both LogCombiner and TreeAnnotator are available with the BEAST V. 2.0.2 software package. Trees were visualised in FigTree V. 1.4. The obtained tree was then utilised as the user-specified species tree required for the Bayesian species delimitation analyses.

To test whether the *P. clarus*/*P. depressus* species complex does indeed comprise three undescribed cryptic lineages as inferred by Daniels *et al*. (2003), we conducted a multilocus coalescent species delimitation analysis implemented in the C-program, Bayesian phylogenetics and phylogeography (BP&P) V. 2.2 (Rannala and Yang 2003; Yang and Rannala 2010). This method accommodates the biological species concept, the species phylogeny represented by a user-specified guide tree (as obtained above with *BEAST) as well as lineage sorting due to ancestral polymorphism (Yang and Rannala 2010). All nodes that had a posterior probability support of 1.0 from the *BEAST topology were taken to represent possible species. A gamma prior $G(2, 1000)$, with a mean of $2/2000 = 0.001$, was used on the population size parameters (θs). The age of the root in the species tree (τ0) was assigned the gamma prior G (2, 1000), while the other divergence time parameters were assigned the Dirichlet prior (Yang and Rannala (2010): Eqn 2). Each analysis of 5×10^5 MCMC generations was run twice from different starting seeds to confirm consistency between runs, with a burn-in period of 5×10^4. Parameter estimates between replicate runs were considered adequate when ESS values were > 200 for all parameters.

Divergence times were estimated in BEAST 2.0.2 (Drummond *et al*. 2002, 2012*a*, 2012*b*; Heled and Drummond 2010) using all loci (12S rRNA, 16S rRNA, 28S rRNA, COI, DecapANT and PEPCK). Only mtDNA trees (12S rRNA and 16S rRNA) were linked for analysis because they are linked on the mitochondrial genome. While phylogenetic dating using shallow

phylogenies and slow evolving or less variable genetic markers has minimal influence on the posterior (Brown and Yang 2010), we still included the nuDNA markers (28S rRNA, DecapANT and PEPCK) under a broad uniform substitution rate prior because there are no accepted mutation rates for these markers. Moreover, no freshwater crab fossils have been recorded in South Africa and no single vicariance event can be used for dating the Afrotropical freshwater crabs (Daniels *et al*. 2006*b*, 2015; Daniels 2011). As such, the mutation rates for 16S rRNA and COI were used to estimate divergence times (Jesse *et al*. 2010; Daniels 2011; Shih *et al*. 2011; Klaus *et al*. 2013), while 12S rRNA, 28S rRNA, DecapANT and PEPCK were estimated around the mutation rates of the two mtDNA markers. The mean mutation rates of these loci have been used for estimating divergence time for decapods, including freshwater crabs (Jesse *et al*. 2010; Daniels 2011; Shih *et al*. 2011; Klaus *et al*. 2013), and were as follows: 2.0×10^{-8} per Myr (with a range of 6.40×10^{-9}–1.42×10^{-8} and a standard deviation (s.d.) of 3.059×10^{-9}) for COI and 1.02×10^{-8} per Myr (with a range of 1.40×10^{-8}–2.60×10^{-8}; s.d. = 1.94×10^{-9}) for 16S rRNA. The input (xml) file was created in BEAUti V. 2.0.2 (Drummond and Rambaut 2007; Drummond *et al*. 2012*a*, 2012*b*). The Yule speciation process and an uncorrelated log-normal relaxed molecular clock model were used with the incorporation of tree uncertainty in the MCMC process to infer divergence times Heled and Drummond (2010). Substitution models (obtained from jModelTest, Table S2) and clock rates were linked for the mtDNA, whereas the nuDNA trees were unlinked. Four MCMC chains were run four times for 200×10^6 iterations, with chains and trees sampled every 20×10^3 generations. An effective sample size (ESS) > 200 for each parameter (visualised in Tracer V. 1.5) verified the convergence of the four combined chains (in LogCombiner V. 2.0) (Nei and Kumar 2000; Drummond *et al*. 2002, 2009, 2012*a*, 2012*b*; Drummond and Rambaut 2007; Tamura *et al*. 2011). A maximum clade credibility tree was computed with mean heights in TreeAnnotator V. 2.0 after 10% of the trees were discarded as burn-in. The divergence time tree was visualised in FigTree V. 1.4 Drummond and Rambaut (2007).

Results

Sequence diversity and population relationships

The basic statistics (number of haplotypes (h), haplotype diversity (*Hd*), number of variable (polymorphic) sites, parsimony informative sites and neutrality tests) for all the gene fragments (12S rRNA, 16S rRNA, COI, 28S rRNA, DecapANT and PEPCK) are shown in Table 2. The COI gene fragment was the most parsimony informative of the mtDNA markers and 12S rRNA was the least informative mtDNA marker. Among the nuDNA markers 28S rRNA was the most parsimony informative while PEPCK was the least parsimony informative genetic marker. These sequences were deposited in GenBank (KU156935–KU156981 were the accession numbers for 12S rRNA; KU156982–KU157031 were the accession numbers for 16S rRNA; KU157032–KU157081 were the accession numbers for COI, KU157082–KU157132 were the accession numbers for 28S rRNA; KU157133–KU157175 were the accession numbers for DecapANT and KU157176–KU157224 were the accession numbers for PEPCK).

Phylogenetic reconstruction using mtDNA (12S rRNA, 16S rRNA and COI)

The Bayesian inference and maximum likelihood (ML) analyses retrieved highly congruent trees, hence only a BI tree is shown and discussed (Fig. 2). Both analyses retrieved seven highly divergent and statistically well supported clades (> 0.95 p$P \geq 75\%$). Clade 1 comprised samples from Ndawana, Monks Cowl (B), Himeville, Coleford, Cobham, Bushmans Nek and Rougham. Clade 2 comprised samples from the Lower Mzumkhulu (B), Cathedral Peak (B), Vergelegen, Vergelegen Nature Reserve, Sani Pass, Sani Pass Hotel, Lower Garden Castele and Garden Castel Nature Reserve. Clade 3 comprised samples from Kamberg, Lower Mzimkhulu (A), Highmoore farm and Highmoore Nature Reserve. The former three clades were sister groups. Clade 4 comprised samples from Gudu falls, Lothoni, Oliviershoek Pass and Mahai (B) sister to clade 5 that comprised of samples from Monks Cowl (A), Highmoore NR and Mahai (A). Clade 6 comprised specimens from Injisuthi (B) and Cathedral Peak (A), while clade 7 was restricted to Injisuthi A. The seven mtDNA clades (Fig. 2), had uncorrected pairwise 'p' COI distances ranging from 4.1% to 15.2% (results not shown), with a mean sequence divergence of 9.81%.

Nuclear DNA (28S rRNA, DecapANT and PEPCK)

Both the BI and the ML tree topologies yielded poorly resolved trees (results not shown). In comparison with the mtDNA, only one identical nuDNA clade was retrieved: clade 4.

Table 2. Basic statistics for the individual genes

Gene fragment	Number of base pairs used[A]/total number of base pairs	Number of Haplotypes	Haplotype diversity	Variable (polymorphic) sites	Parsimony informative sites
12S rRNA	325/353	19	0.933 (s.d. = 0.016)	43	39
16S rRNA	384/536	26	0.940 (s.d. = 0.018)	68	55
COI	451/625	25	0.949 (s.d. = 0.017)	113	107
28S rRNA	420/620	8	0.573 (s.d. = 0.077)	50	46
DecapANT	424/455	11	0.545 (s.d. = 0.091)	20	14
PEPCK	455/493	7	0.272 (s.d. = 0.084)	6	1

[A]Total number of sites (excluding sites with gaps/missing data).

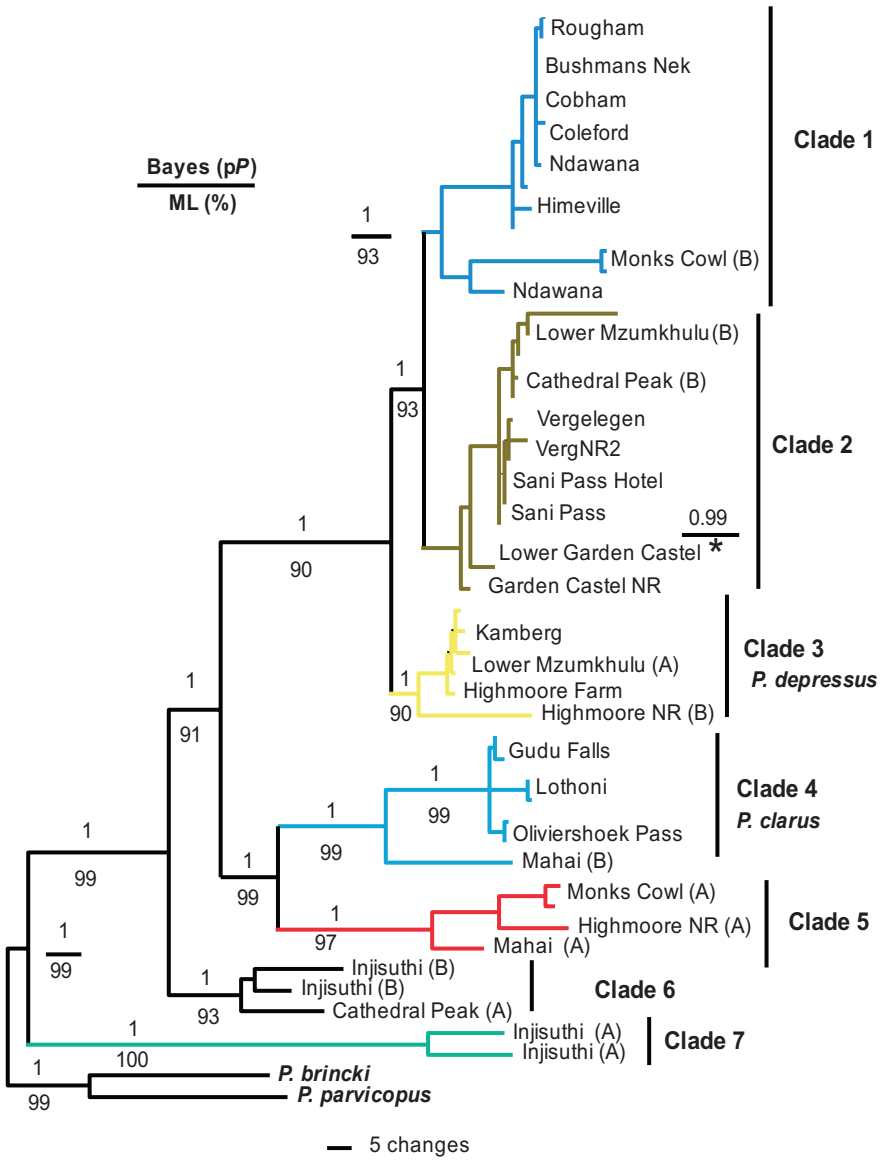

Fig. 2. A combined mtDNA (12S rRNA + 16S rRNA and COI) Bayesian inference phylogram. Values above branches indicate BI posterior probability support (p$P \geq 0.95$), values below branches represent of ML bootstrap support (≥ 75), and an * indicates no support.

Combined mitochondrial and nuclear DNA

The combined DNA sequence dataset (mt and nuDNA) recovered the same seven statistically well supported clades (Fig. 3) evident from the mtDNA topology (Fig. 2). The ML and BI topologies of the combined dataset were congruent hence only the BI topology is showed with very slight exceptions. Three clades in the combined analyses were identical in composition to mtDNA topology (clades 2, 6 and 7; Fig. 2), the remaining four clades had very slight differences in their population composition, and this was generally only confined to one sample locality. Clade 1 comprised specimens from Ndawana, Himeville, Cobham, Coleford, Bushmans Nek and Rougham. In the mtDNA topology (Fig. 2), Monks Cowl (B) was also included in the latter clade. Clade 3 comprised specimens from Lower Mzumkhulu (A), Kamberg and Highmoore farm. In the mtDNA topology (Fig. 2) specimens from Highmoor NR (B) was also included. Clade 4 comprised specimens from Lothoni, Oliviershoek Pass, Gudu Falls, Mahai, Monks Cowl (A) and Highmoore NR. In the mtDNA tree (Fig. 2), the latter clade was only comprised of the first four localities. Clade 5 comprised specimens from Monks Cowl (B) and Highmoore NR (B), while for the mtDNA topology (Fig. 2) it included Mahai (A).

Species tree, species delimitation and divergence time estimation

The species tree suggested eight unique lineages (Fig. 4), corroborating the combined mt/nuDNA dataset (Fig. 3). The species tree obtained from *BEAST with posterior probability

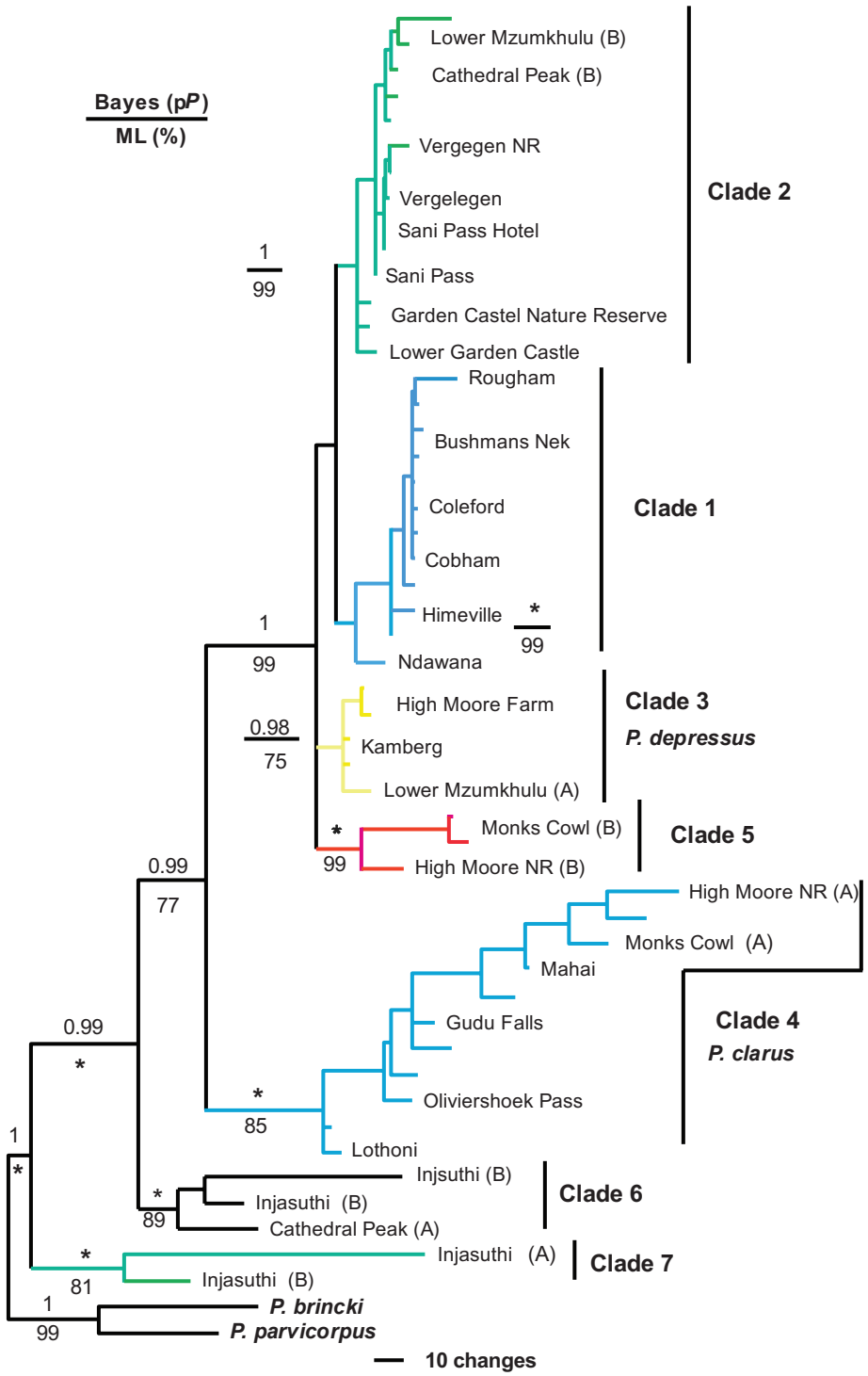

Fig. 3. A combined Bayesian inference phylogram of the combined (mt and nu) DNA sequence dataset (12S rRNA + 16S rRNA + COI + 28S rRNA + DecapANT and PEPCK). Values above branches indicate BI posterior probability support (p*P* ≥0.95), values below branches represent of ML bootstrap support (≥ 75%), and an * indicates no support. The clades were numbered in identical order to the mtDNA topology (Fig. 2).

support of 1.0 for each clade was as follows: (((((((species 8), species 7), species 6), species 5), species 4), species 3), species 1), species 2).

There was support for at least two evolutionary lineages each in Cathedral Peak Injisuthi, Monks Cowl, Highmoore Nature Reserve, Ndawana, Garden Castle Nature Reserve, Kamberg and

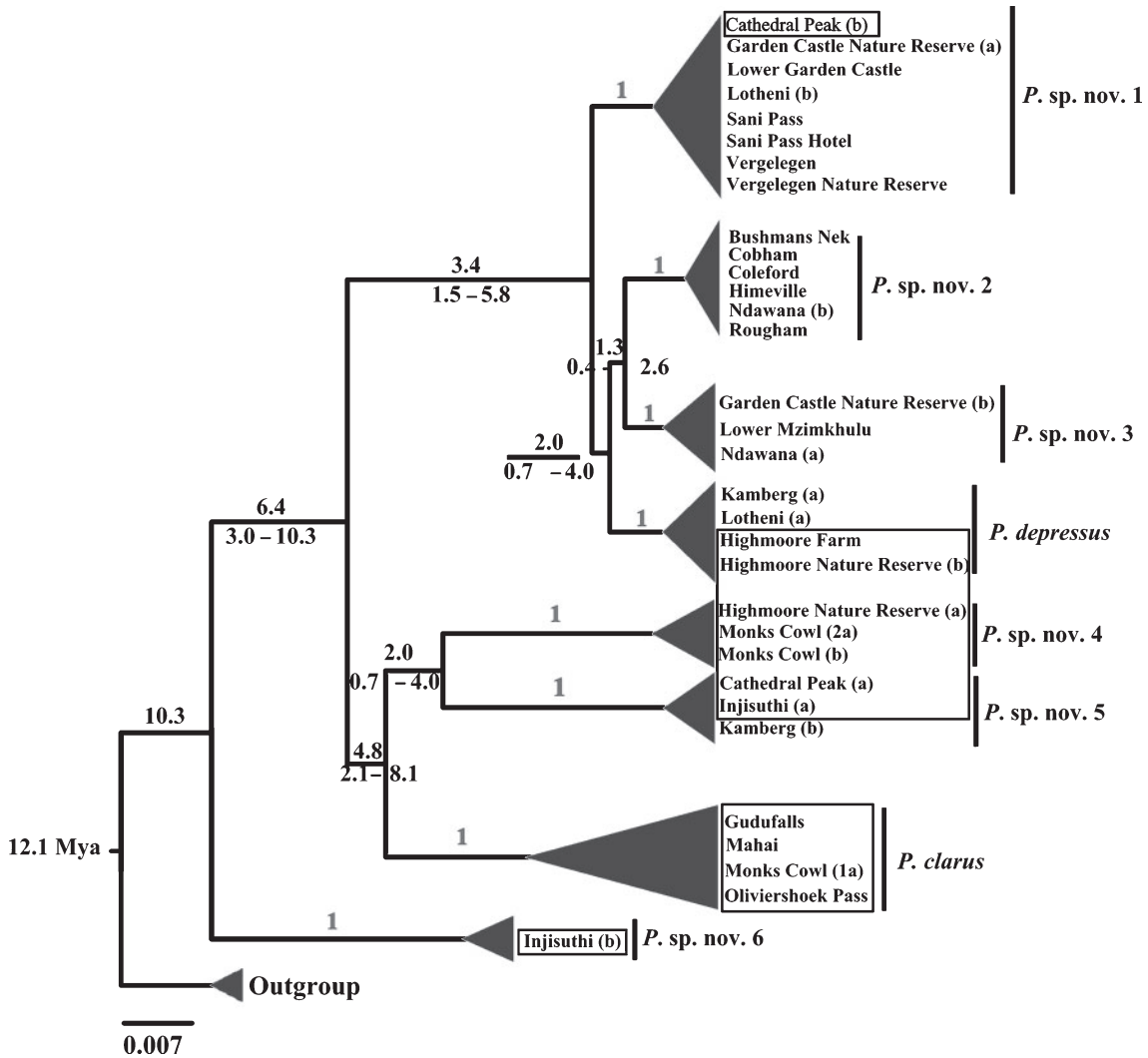

Fig. 4. The *Potamonautes* species tree as given by *BEAST with posterior probability support of 1 (grey values) above the branches. The localities bordered by rectangles are all in the Tugela River drainage, and the rest of the localities are in the uMkomazi River drainage system. On the outer branches, the values above the branches show divergence times of the delimited lineages, and the value below the branches show the 95% higher posterior density (HPD) for each divergence date. Note that although the divergence times for each lineage are shown for the corresponding branches, this tree is not the divergence time tree.

Table 3. Localities comprising sympatric *Potamonautes* lineages based on Fig. 4

Locality	Species
Cathedral Peak (A)	*Potamonautes*, sp. nov. 5
Cathedral Peak (B)	*Potamonautes*, sp. nov. 1
Injisuthi (A)	*Potamonautes*, sp. nov. 5
Injisuthi (B)	*Potamonautes*, sp. nov. 6
Monks Cowl (A)	*P. clarus*
Monks Cowl (B)	*Potamonautes*, sp. nov. 4
Highmoore Nature Reserve (A)	*Potamonautes*, sp. nov. 4
Highmoore Nature Reserve (B)	*P. depressus*
Ndawana (A)	*Potamonautes*, sp. nov. 3
Ndawana (B)	*Potamonautes*, sp. nov. 2
Garden Castle Nature Reserve (A)	*Potamonautes*, sp. nov. 1
Garden Castel Nature Reserve (B)	*Potamonautes*, sp. nov. 3

Lotheni (Figs 3 and 4; Table 3), corroborating the phylogenetic reconstruction results. Thus, 32% of the 25 sampled localities harboured sympatric cryptic lineages. *Potamonautes*, sp. nov. 1 was made up of one locality from the Tugela drainage system (Cathedral Peak) and six localities from the uMkomazi drainage system (Fig. 4). *Potamonautes*, sp. nov. 2 and *Potamonautes*, sp. nov. 3 both comprised specimens from the uMkomazi drainage system. *Potamonautes depressus* occurred in both drainage systems (Kamberg and Lotheni from the uMkomazi and Highmoore from the Tugela drainage systems).

The topology of the species tree (Fig. 4) and divergence time tree (not shown) were congruent. Divergence time estimations revealed that the *P. clarus*/*P. depressus* species complex diverged from its most recent common ancestor between the mid- and late Miocene (12.1 Mya). Though ongoing, divergence within the species complex occurred between *c.* 10.3 Mya,

where *Potamonautes*, sp. nov. 6 diverged from the rest of the species complex, up until *c*. 0.11 Mya in the early Holocene, with the youngest split occurring within *Potamonautes*, sp. nov. 2. Divergence between *Potamonautes*, sp. nov. 1 to *P. clarus* took place ~6.4 Mya (95% highest posterior density (HPD): 3.0–10.3 Mya), separating the Tugela from the uMkomazi drainage populations, with the exception of *P. depressus*, which comprises populations from both drainages. Within the Tugela drainage system, *P. clarus* diverged from the *Potamonautes*, sp. nov. 5/*Potamonautes*, sp. nov. 4 group 4.8 Mya (95% HPD: 2.1–8.1 Mya). The two clades comprising *Potamonautes*, sp. nov. 5 and *Potamonautes*, sp. nov. 4 separated 2.0 Mya (95% HPD: 0.7–4.0 Mya). The remaining four species (*Potamonautes*, sp. nov. 1–*P. depressus*), split from each other 3.4 Mya (95% HPD: 1.5–5.8 Mya), separating *Potamonautes*, sp. nov. 1 from the other three lineages. *Potamonautes depressus* separated from the *Potamonautes*, sp. nov. 3/*Potamonautes*, sp. nov. 2 group ~2.0 Mya (95% HPD: 0.7–4.0 Mya) and *Potamonautes*, sp. nov. 2 diverged from *Potamonautes*, sp. nov. 3 ~1.3 Mya (95% HPD: 0.4–2.6 Mya).

Discussion

This study's novel approach to the delimitation of South African freshwater crab species retrieved eight distinct, though not entirely geographically discrete, lineages within the *P. clarus*/*P. depressus* species complex. Six of these lineages are cryptic and the remaining two clades conform to the two described as *P. clarus* Gouws, Stewart, & Coke, 2000 and *P. depressus* (Kraus, 1843). Together with a more fine scale sampling approach, the use of Bayesian species delimitation methods revealed strong statistical support (posterior probability of 1.00) for eight lineages within the *P. clarus*/*P. depressus* species complex (Fig. 4). Concatenating the data of the six DNA loci sequenced yielded both seven (mtDNA) and seven (combined mt- and nuDNA) possible lineages, with some incongruencies between the gene trees and the species tree (Figs 2 and 3). Incongruencies between concatenated gene and species trees can be attributable to the view that, contrary to the ML and BI methods, the coalescent approach models the ancestral coalescent process as well as unknown gene trees of the lineages independent of mutation (de Queiroz 1998; Edwards 2009; Knowles 2009; Leaché and Fujita 2010; Yang and Rannala 2010; Camargo and Sites 2013; Rannala and Yang 2013). Furthermore, species delimitations undertaken on DNA sequence data (Fig. 4) are prone to overestimation of species thus explaining the differences between the combined DNA sequence topology and the number of retrieved lineages.

Of the six loci used in the present study, PEPCK was found to be the least variable marker, while COI was the most variable and fastest evolving marker. However, protein-coding nuDNA markers such as PEPCK and NaK (Sodium-Potassium ATPase α-subunit) have been suggested as being ideal for phylogenetic delineation among decapods (Tsang *et al*. 2008). While this may be true at the higher-level systematics (Tsang *et al*. 2008), the level of invariability at this locus suggests that nuDNA protein-coding genes may be less suitable for delineating closely related or recently diverged Arthropod lineages.

Cryptic diversity within freshwater crabs is well documented (Daniels *et al*. 1998, 1999, 2003; Jara *et al*. 2003; Jesse *et al*. 2010; Cumberlidge and Daniels 2014; Phiri & Daniels 2014). Therefore, the recovery of six undescribed lineages within the *P. clarus*/*P. depressus* species complex highlights the dubious nature of the taxonomy of species complexes in freshwater crabs. This study supported the monophyly of *P. clarus*, which occurs in the northernmost bounds of the Tugela River drainage system (Gouws *et al*. 2000; Daniels *et al*. 2003). It was previously reported that *P. clarus sensu* stricto occurred in three localities in the northern parts of the Tugela River drainage system (Daniels *et al*. 2003). In the present study we found that the same three populations (i.e. Gudufalls, Mahai and Oliviershoek Pass) maintained their grouping and we added a fourth locality in Monks Cowl (Fig. 4).

Potamonautes clarus was found to be sister to two cryptic sister lineages (*Potamonautes*, sp. nov. 4 and *Potamonautes*, sp. nov. 5, Fig. 4) comprising specimens from Monks Cowl and Highmoore Nature Reserve. *Potamonautes*, sp. nov. 4 is unique to the present study. We also identified two cryptic lineages within Highmoore Nature Reserve, one within *Potamonautes*, sp. nov. 4 and the other within *P. depressus* (Fig. 4). We recovered the same grouping between the sympatric lineage from Cathedral Peak and Injisuthi as per previous study (Daniels *et al*. 2003); however this clade also included specimens from Kamberg (*Potamonautes*, sp. nov. 5, Fig. 4). It is apparent that the specimens from Injisuthi comprises two sympatric lineages, one of which was not identified in the previous study by Daniels *et al*. (2003), and is presented here as a new lineage, *Potamonautes*, sp. nov. 6 (Fig. 4).

Earlier Daniels *et al*. (2003) suggested that *P. depressus* was restricted to Lotheni and Kamberg. However, additional sampling has revealed that the species has a wider distribution and also occurs around Highmoore. This finding also points to a sympatric occurrence of cryptic lineages in Kamberg. Therefore, because the previous study (Daniels *et al*. 2003) sampled fewer localities, cryptic lineage clusters may have been superficial.

Here, we expanded the number of localities of a lineage of a lineage that was previously recognised by Daniels *et al*. (2003) as a sympatric lineage occurring in Cathedral Peak. For example in the combined DNA sequence topology (Fig. 3) specimens form the latter locality is present in both clades 2 and 6. Similarly, Monks Cowl is another locality where sympatric lineages of freshwater crabs occur, *Potamonautes*, sp. nov. 4 and *P. clarus*, (Fig. 4).

Notably, *Potamonautes*, sp. nov. 2 comprised specimens from Coleford, a locality that was also sampled in a previous study (Gouws *et al*. 2000). Previously, Gouws *et al*. (2000) recognised specimens from Coleford as *P. depressus* by assuming that all morphologically identical species occurring south of Kamberg belonged to this species. Specimens from Ndawana (*Potamonautes*, sp. nov. 3), are unique to this study, i.e. *Potamonautes*, sp. nov. 2 is a novel lineage. Therefore, not all lineages that occur in the drainages between Kamberg and Coleford are *P. depressus*. Clearly, the taxonomy of these crabs needs revision *P. depressus* requires redescription and species boundaries need to be re-established. Thus, from the results, we deduce that *P. depressus* only occurs in four localities (Highmoore Farm and Nature Reserve, Kamberg and Lotheni)

and that the specimens from the rest of the localities south of Kamberg and Lotheni (localities 11 and 12, Fig. 1) are separate novel lineages that need to be described.

Therefore, we have recovered an additional three novel lineages (to the three lineages in Daniels *et al*. (2003)) along the Drakensberg Mountain range. Although freshwater crabs have recently been shown to harbour high cryptic diversity (Daniels *et al*. 2003; Jesse *et al*. 2010; Cumberlidge and Daniels 2014; Phiri and Daniels 2014), for southern African freshwater crabs, this level of sympatry has never been recorded. At present, we cannot infer whether these sympatric populations are reproductively isolated, particularly within Injisuthi, where the combined dataset (mt- and nuDNA) phylogenetic reconstructions revealed possible hybridization between the two gene pools. However, this is likely due to historical mixing, rather than present hybridization because both the concatenated mtDNA and concatenated nuDNA did not show this relationship (Fig. 2). On the contrary, sympatry within localities as well as the presence of eight lineages was confirmed by the divergence time estimations results where each lineage had a firm divergence time linked to its speciation (Fig. 4). The divergence time results of between 10.3 and 0.11 Mya should be interpreted with caution because the high 95% HPD range are reflective of the uncertainty of this method.

Nevertheless, the histories of the hydrological network and geological events of the Drakensberg Mountain range are poorly studied. The Drakensberg Mountain range is thought to have formed during the Mesozoic (from the late Triassic to mid-Jurassic, between 240 and 138 Mya) (Fitch and Millar 1971; Dingle *et al*. 1983; Daniels *et al*. 2003). While there is insufficient data available to link the divergence of taxa during the Miocene, it is presumed that the drainage network of the KwaZulu–Natal Province formed during the late Miocene/early Pliocene (Schmitz and Rooyani 1987). For other parts of South Africa, particularly the west, the Miocene/Pliocene is associated with intense upliftment episodes (Partridge and Maud 2000) followed by periods of stasis and erosion (Partridge and Maud 1987; Schmitz and Rooyani 1987; Partridge 1998). In the Cape Fold Mountain range in Western Cape Provinces, South Africa, is well documented that this time period may have been associated with mass cladogenesis (specifically the contraction and expansion of inland taxa), with climate and geological changes being the major drivers of speciation (Partridge and Maud 1987; Daniels *et al*. 2004, 2007; Cowling *et al*. 2008; Swartz *et al*. 2009; Linder *et al*. 2010). These climatic conditions were associated with wet/dry cycles, which may explain the seemingly narrow distribution ranges of some of the species recovered in the present study. There are many isolated headlands as well as detached river valleys within the KwaZulu–Natal drainage network (Rivers-Moore *et al*. 2007). Breaks in the hydrological network could lead to the isolation of lineages because the formed barriers may limit species distribution and faunal exchange between drainages, shaping the patterns of diversification (Hughes and Hughes 1992; Belliard *et al*. 1997; Gascon *et al*. 2000; Cook *et al*. 2002; Rivers-Moore *et al*. 2007; Diedericks and Daniels 2014). Within KwaZulu–Natal, these hydrological breaks are denoted by the presence of waterfalls and hot springs, which may give refuge to endemic taxa (Rivers-Moore *et al*. 2007).

Implications for Conservation

The discovery of additional cryptic lineages within the Drakensberg Mountain range, between the Tugela and uMkomazi drainage systems has conservation implications. We have recognised some lineages that have restricted distribution ranges. Here we confirmed that *P. clarus* is restricted to the northern bounds of the Tugela drainage system as previously recorded (Gouws *et al*. 2000; Daniels *et al*. 2003). This area is maintained by the well managed Ezemvelo KwaZulu–Natal (KZN) Wildlife conservancy and is regarded as one of the high priority freshwater conservation bioregions for conservation in KwaZulu–Natal (Fraser and Keddy 2005). However, conservation planning has only targeted broad-scale biogeographic regions where amphibians and freshwater fish species, particularly the five species of yellow fish, *Labeobarbus* spp., occur. Besides the Odonata (Samways *et al*. 2012), there has been no other study that included aquatic invertebrates for conservation purposes in the region and in South Africa as a whole. In this study, the presence of six novel lineages (three of them novel to this study) suggests that particular attention needs to be afforded to fine-scale water bioregions, within the areas that are managed by the Ezemvelo KZN Wildlife conservancy, mostly within the uKhahlamba Drakensberg National Park. Some of the lineages recovered here (e.g. *Potamonautes*, sp. nov. 6 and *Potamonautes*, sp. nov. 7) seem to have narrow distribution ranges, therefore those specific localities must be considered during future conservation assessments because they may require different conservation strategies. While all the localities fall within the Ezemvelo KZN Wildlife conservancy, Coleford Nature Reserve seemed to be abandoned and was not well managed when we collected our specimens in September 2011 (Phiri, pers. obs.). This could potentially pose a risk to the novel freshwater crab lineage (*Potamonautes*, sp. nov. 2) that is yet to be described. Although the species is present in five other localities, we are uncertain of how wide its distribution range might be, considering that Coleford was the southernmost locality that we sampled. The main conservation recommendation is that the conservation bodies within the province should re-evaluate the freshwater conservation bioregions, especially those identified to harbour the lineages discovered in this study. The need for renewed freshwater bioregion conservation strategies within the Drakensberg Mountain range, and KwaZulu–Natal as a whole, has already been identified (Rivers-Moore *et al*. 2007; Rivers-Moore and Goodman 2010). In a detailed conservation plan (Rivers-Moore *et al*. 2011), it is suggested that in order to properly conserve freshwater diversity, conservation planners will have to consider the spatial correlations across multiple higher taxa, i.e. a finer scale survey coupled with the assessment of genetic diversity as well as the considering the geologic history of the region. Moreover, Rivers-Moore *et al*. (2007) reiterated the importance of surveying the species diversity of invertebrates, and particularly macro-invertebrates, because there is a considerable number of undescribed aquatic invertebrate species (Rivers-Moore *et al*. 2007; Rivers-Moore *et al*. 2011). The present study has provided a starting point by identifying eight freshwater crab lineages that were previously recorded as between two (Gouws *et al*. 2000) and five (Daniels *et al*. 2003) lineages.

Conclusions

This study revealed the presence of multiple, isolated, sometimes sympatric novel lineages of freshwater crab species within the *P. clarus*/*P. depressus* species complex occurring in the Drakensberg Mountain range. Genetic divergences between the recovered lineages were further corroborated by the divergence time estimations. The degree of the underestimation of species diversity among freshwater crabs within this region, and the Afrotropical region, is largely obscured by morphological convergence and limited sampling efforts. In this study, we have shown that there are at least four lineages (including *P. depressus*) between Kamberg and Coleford, but due to a lack of fine-scale sampling, these lineages were previously thought to be one species, i.e. *P. depressus*. This highlights the need for finer scale sampling as well as the more frequent use of molecular data to verify species boundaries, where morphology may be ambiguous. Unfortunately, the current taxonomic placements of many potamonautid species are still largely based on morphological characteristics, which for some species are slowly being proven to be of limited diagnostic value, at least within *Potamonautes* (e.g. Phiri and Daniels 2014). For example, Phiri and Daniels (2014), observed limited variation in gonopods 1 and 2 among three cryptic species recovered from the *P. perlatus* species complex. We suggest that species boundaries should be evaluated in relation to the morphology, genetic data and ecological boundaries (Padial and de la Riva 2009) as well as the geological timescale. The morphological description of the six novel *Potamonautes* species delimited here is beyond the scope of this study and will be conducted at a later stage following the addition of more sampling localities.

Here, we focussed on the highland areas of the Drakensberg Mountain range in KwaZulu–Natal Province. There remains uncertainty regarding the level of cryptic diversity in the freshwater crabs found in this province. Although South Africa is the most comprehensively sampled country for freshwater crab diversity relative to other African countries, the discovery of six new species of freshwater crabs here suggests that it is highly probable that continued sampling will retrieve more novel freshwater crab species, particularly in low-lying areas of the KwaZulu–Natal Province. As the number of novel and/or cryptic lineages increases, so do the implications for conservation, particularly because undiscovered cryptic lineages may already be facing extinction or habitat destruction.

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