Molecular phylogeny and biogeography of the Afrotropical freshwater crab fauna

by

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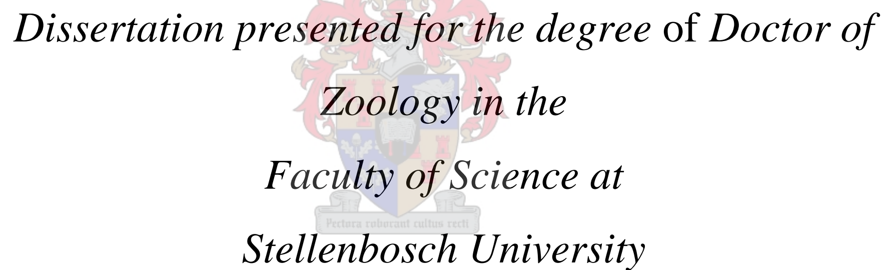

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DECLARATION

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ABSTRACT

Freshwater organisms, such as crabs (Crustacea: Decapoda: Brachyura), are useful in studies examining inland historical biogeographic patterns and speciation because they are isolated to specific drainage systems, which often serve as barriers to gene flow. The Afrotropical freshwater crab fauna (Potamonautidae) present ideal organisms for investigating hypothesis relating to evolutionary histories because they occur on continental Africa (sub-Sahara) and islands. However, there is a great deal of undiscovered freshwater crab diversity, especially with the prevalence of undiscovered cryptic lineages, which are poorly studied among freshwater crabs, leading to uncertain regional diversity.

In this research, multiple genetic (mt- and nuDNA) markers were used to infer the phylogenetic relationships and the biogeographical histories of the Afrotropical freshwater crab superfamily, Potamonautidae. Divergence time estimations were used to infer biogeographic histories, to ascertain whether speciation could be linked to past geologic and / or climatic events.

Two widely distributed *Potamonautes* species complexes were targeted for the investigation of regional cryptic species diversity. In Chapter 2, the intraspecific phylogenetic variability within *Potamonautes perlatus* sensu lato occurring on the Cape Fold Mountain range (South Africa) was examined, with sampling localities occurring in western- and southern flowing drainages. Previous research suggested possible cryptic speciation within this species complex; however, no tangible inferences could be made because of analytical constraints. Two major clades were recovered: one corresponding to western flowing drainages and another to southern flowing drainages. Moreover, three cryptic lineages were recovered: *P. perlatus* sensu stricto, restricted to western flowing drainages, and two geographically discrete novel cryptic lineages from the southern flowing drainages, described as *P. barbarai* sp. nov and *P. barnardi* sp. nov., with divergence (±2.61 Mya) linked to Pleistocene climatic events.

Subsequent to the recovery of the two novel lineages from the Cape Fold Mountain range, the revision of the *P. clarus* / *P. depressus* species complex from the Tugela and uMkomazi drainages (Drakensberg Mountain range, South Africa) was conducted. This species complex was previously found to comprise at least five cryptic lineages (Chapter 3). A coalescent multilocus (three mt- and three nuDNA) Bayesian species delimitation method was used, and an additional three cryptic lineages were recovered, bringing the total to eight

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species (two already described as *P. clarus* and *P. depressus*), with divergence having occurred approximately 10.3 Mya.

Following the recent discovery of novel freshwater crab lineages in the mountainous areas of Mozambique and Malawi, a sampling trip to the Zimbabwean Highlands was undertaken, where a novel freshwater crab species was discovered and described as *P. mutareensis*, highlighting the need to sample high-lying regions (Chapter 4). Furthermore, two additional novel lineages from Mozambique (*P. bellarussus* sp. nov.) and the Mpumalanga Province in South Africa (*P. flavusjo* sp. nov) were described (Chapter 5).

In Chapter 6, increased taxon sampling, with additional specimens acquired from various museums and personal collections was used to obtain a better resolution of the phylogeny of the Afrotropical Potamonautidae and to infer the ancestral affinities of the two sub-families, Deckeniinae and Potamonautinae. The Potamonautidae were found to have speciated eastward from West Africa, with a late Cretaceous divergence (±107 – 96.04 Mya). The Potamonautinae originated in West Africa (three genera), while the paraphyletic *Potamonautes* and *Platythelphusa* had East African affinities. *Potamonautes* was not monophyletic, comprising several fragmented geographic clades, which may suggest that this genus requires revision. Nevertheless, the overall speciation within the Potamonautidae reflects past geological and climatic events, such as rifting and uplift episodes and the contraction of forests, which occurred from the Tertiary onwards.

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On the eve of submitting this thesis, just before midnight, it was announced that Nelson Mandela had died and I was reminded of what he said in his book *Long Walk to Freedom*:

– I have walked that long road to freedom. I have tried not to falter; I have made missteps along the way. But I have discovered the secret that after climbing a great hill, one only finds that there are many more hills to climb. I have taken a moment to have a rest, to steal a view of the glorious vista that surrounds me, to look back on the distance I have come. But I can only rest for a moment, for with freedom come responsibilities, and I dare not linger, for my long walk has not ended. –

R.I.P. N.R.M. – 05/12/2013

These words rang true for me in the days following his death. I reflected on how hard I'd fought to get to where I am today and it all culminates to this one moment. I would like to thank everyone that helped me during this last month of the PhD (November 2013). Without the following people I probably would not have been able to complete this thesis in time. So thank you to Candice, Genevieve, and Nuraan for encouraging me through my long walk, making sure I don't falter:

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CHAPTER 1

GENERAL INTRODUCTION

Primary freshwater crabs (Crustacea: Decapoda: Brachyura Linnaeus, 1758) are a diverse assemblage of eubrachyurans, representing approximately 25% of Brachyuran diversity, comprising two superfamilies (Potamoidea Ortmann, 1896 and Trichodactyloidea H. Milne Edwards, 1853 (Cumberlidge & Ng, 2009). Globally, over 1300 freshwater crab species have been described (Yeo *et al*., 2008), with novel operational taxonomic units being added constantly. Freshwater crabs have a pantropical distribution in the inland waters of the continents and adjacent islands throughout the tropics and subtropics (occurring in Neotropical, Palaearctic, Oriental, Australasian, and Afrotropical regions) (Yeo *et al*., 2008). Freshwater crabs are found in virtually all types of freshwater bodies including fast-flowing rivers, swamps, ponds, rice fields, freshwater deposits in tree holes, and mud burrows from both low-lying altitudes to high mountainous areas on continents and continental islands, and some exceptional instances on volcanic islands (for example, Silhouette Island, Seychelles) (Sternberg & Cumberlidge, 2001; Yeo *et al*., 2008; Cumberlidge & Ng, 2009).

Freshwater crabs are characterized by the production of large yolk-rich eggs, the absence of a dispersive larval phase (termed direct development, though they have been recently been reported to undergo larval development inside the egg (Xue *et al*., 2013), the completion of their life cycle in freshwater bodies, and their inability to survive prolonged exposure to marine environments. They are among the largest macro-invertebrate detritivores (Ng & Yeo, 2013) in freshwater ecosystems where they play a critical role in ecological structure and function (Dobson, 2004; Dobson *et al*., 2007). They reduce leaf litter particle size, which is important for microbial activity, thereby providing nutrition for filter-feeding aquatic fauna (Hill & O'Keeffe, 1992; Dobson *et al*., 2002, 2007; Dobson, 2004). Together with their ubiquity and high biomass in inland aquatic ecosystems of tropical regions, the role they fulfil renders them important contributors to the dynamics of nutrient recycling in rivers (Hill & O'Keeffe, 1992; Dobson *et al*., 2002).

Moreover, freshwater crabs are an important food source for a large number of freshwater predators (Turnbull-Kemp, 1960; Arkell, 1979; Purves *et al*., 1991; Butler & Marshall, 1996; Cumberlidge *et al*., 1999). Apart from being consumed by humans for subsistence, freshwater crabs are important for supporting small commercial fisheries, with some species serving as a food source for a variety of commercially important fish species (Udonsi, 1987; Sachs & Cumberlidge, 1991; Cumberlidge *et al*., 1999; Cumberlidge *et al*.,

2009). Furthermore, because of their vulnerability to disturbances such as pollution, freshwater crabs have also been used as indicators for freshwater quality (Sanders *et al*., 1998; Steenkamp *et al*., 1993; Thawley *et al*., 2004; Gagneten *et al*., 2012).

In addition to their ecological and economic importance, freshwater crabs can also provide insight into the evolutionary histories of inland systems, such as rivers and lakes, which may be useful in examining biogeographical and hydrographical hypotheses (Ng & Rodriguez, 1995; Daniels *et al*., 2002; Daniels, 2003; Daniels *et al*., 2006a; Shih *et al*., 2006). However, their use as evolutionary models has been hampered by the lack of well-resolved phylogenies among freshwater decapods families (Daniels *et al*., 2002; Perez-Losada *et al*., 2002; Munasinghe *et al*., 2004). Furthermore, the phylogenetic relationships among the global freshwater crab fauna (superfamilies and families) remains relatively unexplored, with systematic and biogeographic studies from most regions being absent. Moreover, in some areas such as the Afrotropical region (sub-Saharan Africa and major adjacent islands, Madagascar, Seychelles, and Socotra), particularly on continental Africa, sampling is incomplete, especially in areas that have been recognized as centres of diversity for freshwater crabs. Freshwater crabs are thus greatly in need of a comprehensive taxonomic revision that more accurately reflects their evolutionary relationships.

TAXONOMY

There is a great deal of uncertainty in the taxonomic assignments and classifications of the global freshwater crab fauna, and specifically in the Afrotropical region, although significant strides have been made in recent years (Cumberlidge *et al*., 2008; De Grave *et al*., 2009). This uncertainty is attributable to the historical use of morphological characters in the taxonomy of freshwater crabs (Bott, 1970a; Guinot *et al*., 1997; Stewart, 1997a; Cumberlidge, 1999; Martin & Davis, 2001; Cumberlidge & Sternberg, 2002; Daniels *et al*., 2006b; Klaus *et al*., 2006; Cumberlidge *et al*., 2008; Ng *et al*., 2008; Cumberlidge & Ng, 2009). However, the recent use of molecular phylogenetic analyses has revealed some significant conflicts (Daniels *et al*., 2002, 2006b), indicating that current taxonomic assignments of freshwater crabs may be representing flawed taxonomic groupings. Nevertheless, there have been instances where morphology has provided partial support for molecular-based classifications (e.g. Klaus *et al*., 2009). Nonetheless, the consequence of uncertainties in their classification is notable at the higher taxonomic levels of assignment (Table 1).

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These uncertainties are detailed in Cumberlidge *et al*. (2008). Briefly, Bott (1970) classified freshwater crabs into five superfamilies: Pseudothelphusoidea Ortmann, 1853, Potamoidea Ortmann, 1896, Deckeniidae Ortmann, 1897, Gecarcinucoidea Rathbun, 1904, and Portunoidea Rafinesque, 1815, comprising 11 families (Table 1). This classification was supported by Cumberlidge (1999). However, Martin & Davis (2001) reduced the number of families from 11 to seven. A significant adjustment in the higher classification of freshwater crabs at the superfamily level took place when Cumberlidge *et al*. (2008) and Ng *et al*. (2008) recognized Trichodactyloidea H. Milne Edwards, 1853 as a superfamily (family: Trichodactylidae H. Milne Edwards, 1853) and dissolved the Portunoidea Rafinesque, 1815 (family: Trichodactylidae H. Milne Edwards, 1853). Cumberlidge & Ng (2009) further dissipated two superfamilies (Pseudothelphusoidea Ortmann, 1853 and Gecarcinucoidea Rathbun, 1904), resulting in the paraphyletic relationship between two distinct monophyletic lineages: the Trichodactyloidea H. Milne Edwards, 1853 (with one family) and the Potamoidea Ortmann, 1896 (with four families) as superfamilies. The use of molecular phylogenetic methods to identify and classify species can overcome some of these issues and can be used to establish evolutionary relationships and patterns of diversity that reflect natural groupings.

However, freshwater crabs have received less attention in phylogenetic studies compared to their marine counterparts, with only a few attempts at identifying relationships between freshwater crabs and other brachyurans found in marine environments (Sternberg *et al*., 1999). Despite a number of morphological cladistic studies (Cumberlidge, 1999; Cumberlidge & Sternberg, 1999a, b; Sternberg & Cumberlidge, 1999; Sternberg *et al*., 1999), the phylogenetic affinities of freshwater crabs remain unclear. Consequently, there is still no consensus on the validity of the higher-level taxonomic classification of freshwater crabs, with some studies suggesting that the taxonomic placement of the five families may be artificial (Daniels *et al*., 2006a; Klaus *et al*., 2006; Cumberlidge *et al*., 2008; Yeo *et al*., 2008; Cumberlidge & Ng, 2009; Klaus *et al*., 2009).

TAXONOMY OF AFROTROPICAL FRESHWATER CRABS

Currently, over 145 species of freshwater crabs are known to occur in the Afrotropical region and are classified into two families (Cumberlidge & Ng, 2009). The family Potamidae Ortmann 1896 is restricted to North Africa (*Potamon* sp.) and Socotra Island (two endemic genera comprising three species: *Socotra* and *Socotrapotamon*) (Cumberlidge, 1999;

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Cumberlidge, 2008; Yeo *et al*., 2008; Cumberlidge *et al*., 2009; Cumberlidge & Ng, 2009). The second and dominant endemic family occurring in the Afrotropical region is the Potamonautidae (Bott 1970a). This family occurs in sub-Saharan Africa as well as volcanic islands in the Atlantic Ocean (such as Principe and Sao Tome) and island in the Indian Ocean, i.e. Seychelles, Zanzibar, Pemba and Mafia, and Madagascar (Yeo *et al*., 2007; Cumberlidge, 2008; Cumberlidge & Ng, 2009).

The morphology-based taxonomic assignments of Afrotropical freshwater crabs are largely based on Bott's (1955) monographic work on the African freshwater crabs where he assigned three continental genera *Potamonautes*, *Sudanonautes* and *Liberonautes* to Potamonidae. As summarised in Cumberlidge *et al*. (2008), *Platythelphusa* and *Erimetopus* were assigned as sub-genera of *Potamonautes*, and the Malagasy taxa were assigned to Hydrothelphusinae. According to Bott (1959, 1960, 1965, 1970a, b) there were four families and three sub-families in Africa and Madagascar: the Potamonautidae, Potamidae, Deckeniidae, and Gecarcinucidae. Although, Bott's taxonomic assignments and classifications have profoundly influenced present-day systematic arrangements of the Afrotropical freshwater crabs, not all taxonomists accept his conclusions. Bott's (1965) revision of the Malagasy freshwater crabs demonstrated an inconsistent morphological character application, specifically the mandibular palp, when he assigned three genera to three sub-families: *Madagapotamon* (simple terminal segment, Potamoninae), *Gecarcinautes* (simple terminal segment with a small basal ledge counted by Bott (1965) as bilobed, Gecarcinucinae), and *Hydrothelphusa* (bilobed terminal segment, Hydrothelphusinae). *Hydrothelphusa* and *Madagapotamon* were considered to have African affinities, and *Gecarcinautes* to have affinities with West Africa, South Africa, and India, thereby inferring a close phylogenetic relationship between the crabs of these regions. Bott (1955) further demonstrated similar inconsistent morphological applications when he assigned the Seychelles' freshwater crab to *Deckenia* (Deckeniidae), despite differences in its mandible and that of *Deckenia* from East Africa. These two groups have now been found to have a sister relationship, although traditionally placed in different families and superfamilies (Ng *et al*., 1995; Stevcic, 2005). The genera *Seychellum* and *Deckenia* now assigned to the same family (Potamonautidae) and sub-family (Deckeniinae) (Daniels *et al*., 2006b; Cumberlidge *et al*., 2008; Klaus *et al*., 2009).

Nonetheless, more taxonomic revisions are gradually becoming available for the freshwater crabs of some parts of Africa (Marijnissen *et al*., 2004; Cumberlidge & Tavares, 2006; Daniels *et al*., 2002, 2006b). Recent taxonomic endeavours aided by the use of

molecular phylogenetic analyses have unravelled some evolutionary relationships among the Afrotropical freshwater crab fauna. One such example is the reassignment of the Afrotropical Gecarcinucidae of Bott (1970a), which has been impacted by several of these taxonomic contributions. Ng *et al*. (1995) transferred *Seychellum* from the Deckeniidae to the Gecarcinucidae, while both Cumberlidge (1996a, b) and Stewart (1997a, b) independently transferred *Gecarcinautes brincki* from the Gecarcinucidae to *Potamonautes* in the Potamonautidae, sinking the genus *Gecarcinautes* for the African continent (Cumberlidge & Sternberg, 2002). Moreover, Cumberlidge (1999) transferred three West African genera (*Afrithelphusa*, *Louisea*, and *Globonautes*) from the Gercarcinucidae to the Globonautinae; and Cumberlidge & Sternberg (2002) assigned the entire Malagasy fauna to the Potamonautidae and recognized no Gecarcinucid genera on the island. As such, there are no known Gecarcinucidae on the African continent or Madagascar. These findings suggest that interpretations of morphological character states, which are based on subjective observations, can lead to family-level errors of assignment.

Similarly, the Potamonautidae have also been impacted by taxonomic contributions that have cast doubt on the 15 sub-genera proposed by Bott (1955) (Cumberlidge, 1999; Cumberlidge *et al*., 1999). For example, Daniels *et al*. (2002) demonstrated that southern African *Potamonautes* species were inconsistent with Bott's (1955) sub-generic designations. While Bott (1955) assigned *P. depressus* and *P. sidneyi* to the sub-genus *Orthopotamonautes*, Daniels *et al*. (2002) found that these two species were not sister taxa. In another example, Cumberlidge & Reed (2004) transferred the Central African sub-genus *Potamonautes* (*Erimetopus*) Bott, 1955 to *Erimetopus*. *Potamonautes* (*Platythelphusa*) Bott, 1955 was transferred to *Platythelphusa*, and this genus was transferred from the Potamonautidae to the Platythelphusidae (Cumberlidge, 1999; Cumberlidge *et al*., 1999). The most recent taxonomic revision of the Afrotropical freshwater crabs was conducted by Cumberlidge *et al*. (2008) and De Grave *et al*. (2009) where they provided some level of resolution to relationships within the Afrotropical freshwater crabs. However, the most recent taxonomic classifications (Cumberlidge *et al*., 2008; De Grave *et al*., 2009) highlight the uncertainty regarding the generic placements within sub-families (Table 2) because there is disagreement on the number of sub-families and the placement of genera within the Potamonautidae (see Table 2). Moreover, it is perceptible that some of the widespread species may potentially be representative of cryptic lineages (defined here as two or more species that have been classified as a single species because they are comparatively morphologically indistinguishable) (Bickford *et al*., 2007).

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CRYPTIC SPECIATION

There is ample evidence to suggest that a number of widely distributed freshwater crab species in southern Africa are harbouring cryptic diversity (Daniels *et al*., 1998; Daniels *et al*., 2003; Chapter 3, this thesis; Cumberlidge & Daniels, 2014; Phiri & Daniels, 2014). Some freshwater crab species are point endemics with limited distributions (Cumberlidge, 1999; Cumberlidge & Daniels, 2008; Collen *et al*., 2014). However, recent assessments of the Malagasy and southern African suggest that freshwater crab species with restricted distributions are likely to be threatened, vulnerable or endangered (Cumberlidge *et al*., 2002; Cumberlidge, 1999; Dobson, 2004; Cumberlidge & Daniels, 2008; Cumberlidge *et al*., 2009). Extrapolation of localized regional studies to the entire Afrotropical region indicates that it is likely that a large number of freshwater crab species, including as yet unidentified cryptic lineages, may already be vulnerable to extinction particularly under scenarios predicted by climate change.

However, the distribution (and level of cryptic speciation) of many Afrotropical freshwater crab species is still, to a very large extent, unknown. This is because most of the early collectors of freshwater crab specimens provided no maps of the distribution of many species that were historically collected from the Afrotropical region. Additionally, those early collections were sparse and vague, and depended on incorrect identifications and classifications (Cumberlidge, 1999). Furthermore, the systematics of freshwater crabs as a whole, and especially the Afrotropical freshwater crabs, have seldom been studied within an evolutionary context and early assignments were constructed with no indication of genealogical relationships (Cumberlidge, 1999).

There is, nonetheless, evidence that some of widespread *Potamonautes* species display morphological (Barnard, 1950; Daniels *et al*., 1998) and genetic (Daniels *et al*., 1999, 2001, 2003, 2006b) variations. For instance, some forms of *Potamonautes perlatus* (widespread species in southern Africa) are morphologically similar to *P. sidneyi*. In another example, *P. clarus* and *P. depressus* are also known to be morphologically similar, but the use of molecular techniques revealed several distinct clades among populations of these species (Daniels *et al*., 2003). Daniels *et al*. (2003) found that divergence between the different lineages occurred during the late Miocene and early Pliocene, suggesting that accurately delimiting species can also be useful in historical biogeographical studies. However, research on cryptic speciation in freshwater crab diversity is limited. In addition, early studies on species delimitations of Afrotropical freshwater crab diversity only used

enzyme electrophoreses and mitochondrial DNA sequencing, which are now considered unreliable (Song *et al*., 2008; Chu *et al*., 2009). Thus, the existing inventory of freshwater crab diversity may be an underestimate, especially if more cryptic species are present within the current estimate of ±145 in the Afrotropical region.

BIOGEOGRAPHY

Freshwater crabs are an excellent choice for studying historical biogeography hypotheses because of their inability to cross saltwater barriers, their lack of dispersive larval stages, and that they are considered to be drainage basin specific (i.e. populations show strong phylogeographical structuring, which is indicative of low dispersal), with limited evidence for long distance transoceanic dispersal (Morris & van Aardt, 1998; Daniels *et al*., 2002; Daniels, 2003; Sternberg & Cumberlidge, 2001; Shih *et al*., 2006, 2007; Yeo *et al*., 2007; Cumberlidge, 2008). It has, however historically been argued that when compared to other taxa, freshwater crabs are not useful organisms for studies investigating historical biogeography (Banarescu, 1990).

Banarescu (1990) claimed that freshwater crabs were able to cross salt-water barriers, and that they were a product of isolated marine ancestors. Furthermore, Baranescu (1990) postulated that because freshwater crabs could survive outside water and could also disperse for short distances over terrestrial barriers, they were less restricted to their drainage systems. His assumptions were based on the occurrence of the Pseudothelphusidae in the Antilles and on the mainland. The idea of transoceanic dispersal has, however, previously been disregarded because saltwater is regarded as an efficient barrier to dispersal. For instance, Ng & Rodriguez (1995) argued against the transoceanic dispersal hypothesis and suggested that an alternative vicariant hypothesis had more factual support for the current biogeographic patterns for freshwater crabs. They also argued that because of the limited dispersal capacities of freshwater crabs, most species have specific habitat requirements and limited distribution ranges (Ng & Rodriguez, 1995). Instead, mid-Cretaceous vicariant events associated with the breakup of Gondwana were proposed as possible valid hypotheses (Ng & Rodriguez, 1995). However, based on molecular evidence, the Gondwanan ancestry and Indian affinities for the freshwater crabs occurring in Madagascar and the Seychelles were found not to correspond with the geological history of the Madagascar-Seychelles-India split from Africa in the mid-Cretaceous (160 Mya) (Daniels *et al*., 2006b; Cumberlidge, 2008; Klaus *et al*., 2011). As such, several recent molecular phylogenetic studies on freshwater and other taxa advocate

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that transoceanic dispersal is widespread (Daniels *et al*, 2006b; Cumberlidge, 2008; Townsend *et al*., 2010; Daniels, 2011, Esser & Cumberlidge 2011).

Nonetheless, many phylogenetic studies on the biogeography of Afrotropical freshwater crabs have mainly been regional: Western Cape Province of South Africa (Daniels, 1999 a, b; Daniels, 2003; Daniels *et al*., 2006a), Seychelles (Daniels, 2011), East Africa (Marijnissen *et al*., 2006), with a single study that considered the Afrotropical region in its entirety (Daniels *et al*., 2006b). Although the most comprehensive study to date, Daniels *et al*. (2006b) lacked a number of genera (e.g. *Globonautes*, *Afrothelphusa, Louisea* and *Erimetopus*), represented a fraction of the described *Potamonautes*, *Liberonautes*, *Sudanonautes*, and *Platythelphusa* species, and none of the recent discovered diversity.

Therefore, the biogeographical relationships of Afrotropical freshwater crabs are in need of revision, especially if these relationships are to be linked to accurate taxonomic classifications.

AIMS AND RATIONALE FOR THE RESEARCH

The main aim of this research was to re-examine the phylogeny of freshwater crab species (Potamonautidae) occurring in the Afrotropical region using multiple genetic (mitochondrial and nuclear DNA) markers in order to infer their evolutionary histories and biogeographical boundaries. It has been speculated that widely distributed freshwater crabs species in Southern Africa harbour cryptic lineages (Daniels, 2003; Daniels *et al*., 2006a). In order to test this, I examined the phylogenetic relationships among three widespread *Potamonautes* species, i.e. *P. perlatus* (Chapter 2: Phiri & Daniels, 2014) and the *P. clarus* / *P. depressus* species complex (Chapter 3), occurring in South Africa. I expected to find widespread cryptic speciation among the freshwater crab species with wide distribution ranges, potentially harbouring several undescribed species. Specifically, in Chapter 2, I used a combination of mtDNA (16S rRNA and COI) and nuDNA (28S rRNA) loci to examine the intraspecific variability among geographically isolated populations of *P. perlatus* sensu lato from six major drainage systems of the Cape Fold Mountain range (Western Cape Province, South Africa). This particular species complex was chosen because it is widespread and previous research (Daniels, 2003; Daniels *et al*., 2006a) already suggested that at least two cryptic lineages were present, but small sample sizes and the use of outdated analytical methods prevented any firm conclusions. I subsequently described two novel freshwater crab species and used divergence time estimations to determine past climatological or geological

events that may have been instrumental at shaping speciation within *P. perlatus* s.l. (Phiri & Daniels, 2014). Moreover, I used a recently developed Bayesian species delimitation method to confirm the validity of the presence of three undescribed cryptic lineages within the *P. clarus* / *P. depressus* species complex (Daniels *et al*., 2003), which is widespread on the Drakensberg Mountain range (KwaZulu-Natal Province, South Africa) (Chapter 3). I also considered the evolutionary history of this species complex by conducting divergence time estimations to establish the geological time scale period that coincides with the divergence of the recovered lineages.

Furthermore, mountainous areas are poorly sampled, and Daniels & Bayliss (2012) hypothesised that these areas may be harbouring undescribed species diversity and suggested that these regions should be targeted in future sampling endeavours. Following the recent discovery of two novel lineages from Mozambique and Malawi, I visited neighbouring Zimbabwe's East African Highlands, which is poorly sampled with no known endemic freshwater crab fauna. Extensive sampling across the Highlands led to the discovery and description of another novel freshwater crab species (Phiri & Daniels, 2013: Chapter 4). During the course of this research I received specimens from various sources. Jointly, my supervisor SR Daniels (morphology) and I (molecular techniques) identified a novel species (sent to us by J Bayliss) from mountainous areas in Mozambique (Daniels *et al*., submitted: Chapter 5). Also within Chapter 5, another novel freshwater crab species was described from the Mpumalanga Province (South Africa).

Through increased taxon sampling, including the addition of previously excluded genera, I provided a better resolution to the phylogeny of the Potamonautidae and explored their biogeographical affinities (Chapter 6). This study was carried out because the currently comprehensive study was incomplete and taxon poor, and excluded some representatives from key African regions (Daniels *et al*., 2006b) and there is evidence that relationships among Afrotropical freshwater crab genera could be representative of unreliable and flawed taxonomic sub-divisions (Daniels *et al*., 2006a; Cumberlidge *et al*., 2008; De Grave *et al*., 2009).

Finally, the research presented in this thesis was synthesized and put into perspective in Chapter 7.

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Table 1 Changes in the higher taxonomy: superfamilies (in bold) and families of the primary freshwater crabs (Cumberlidge & Ng, 2009).

Bott (1970a) and Cumberlidge (1999)	Martin & Davis (2001)	Cumberlidge *et al*. (2008) and Ng *et al*. (2008)	Cumberlidge & Ng (2009)
Pseudothelphusoidea Ortmann, 1853	**Pseudothelphusoidea Ortmann, 1853**	**Pseudothelphusoidea Ortmann ,1853**	
Pseudothelphusidae Rathbun, 1893	Pseudothelphusidae Rathbun, 1893	Pseudothelphusidae Rathbun, 1893	
Potamocarcinidae Ortmann, 1896			
Potamoidea Ortmann, 1896	**Potamoidea Ortmann, 1896**	**Potamoidea Ortmann, 1896**	**Potamoidea Ortmann, 1896**
Potamidae Ortmann, 1896	Potamidae Ortmann, 1896	Potamidae Ortmann, 1896	Pseudothelphusidae Rathbun, 1893
Potamonautidae Bott, 1970b	Potamonautidae Bott, 1970b	Potamonautidae Bott, 1970b	Potamidae Ortmann, 1896
Deckeniidae Ortmann, 1897	**Deckeniidae Ortmann, 1897**		Potamonautidae Bott, 1970b
Sinopotamidae Bott, 1970a	Platythelphusidae Colosi, 1920		Gecarcinucidae Rathbun, 1904
Isolapotamidae Bott, 1970a			
Gecarcinucoidea Rathbun, 1904	**Gecarcinucoidea Rathbun, 1904**	**Gecarcinucoidea Rathbun ,1904**	
Gecarcinucidae Rathbun, 1904	Gecarcinucidae Rathbun, 1904	Gecarcinucidae Rathbun, 1904	
Parathelphusidae Alcock, 1910	Parathelphusidae Alcock, 1910	Parathelphusidae Alcock, 1910	
Sundathelphusidae Bott, 1969			
Portunoidea Rafinesque, 1815	**Portunoidea Rafinesque, 1815**		
Trichodactylidae H. Milne Edwards, 1853	Trichodactylidae H. Milne Edwards, 1853		
		Trichodactyloidea H. Milne Edwards, 1853	**Trichodactyloidea H. Milne Edwards, 1853**
		Trichodactylidae H.Milne Edwards, 1853	Trichodactylidae H.Milne Edwards, 1853

Table 2 Potamonautidae: sub-families and the placement of extant genera within the sub-families. [1]Additional subfamily and [2] placement of genus within a sub-family by De Grave *et al*. (2009).

Sub-family (number of species)	Placement of genera (Cumberlidge *et al*., 2008)	Placement of genera (De Grave *et al*., 2009)
Potamonautinae Bott, 1970a (122)	*Erimetopus* Rathbun, 1894	*Erimetopus*
		[2] *Foza* Dai & Bo, 1994
	Liberonautes Bott, 1955	*Liberonautes*
	Louisea Cumberlidge, 1994	
	Potamonautes Bott, 1970a	*Potamonautes* MacLeay, 1838
	Potamonemus Cumberlidge & Clark, 1992	*Potamonemus*
	Sudanonautes Bott, 1955	*Sudanonautes*
	Platythelphusa A. Milne-Edwards, 1887	*Platythelphusa*
Deckeniinae Ortmann, 1897 (10)		
Deckeniini Ortmann, 1897 (5)	*Deckenia* Hilgendorf, 1869	*Deckenia*
	Seychellum Ng, Števčić & Pretzmann, 1995	*Seychellum*
Globonautina Bott, 1969 (5)	*Globonautes* Bott, 1959	
	Afrithelphusa Bott, 1969	
Hydrothelphusini Bott, 1955 (14)	*Boreathelphusa* Cumberlidge & Sternberg, 2002	
	Foza Reed & Cumberlidge, 2006	
	Hydrothelphusa A. Milne-Edwards, 1872	
	Madagapotamon Bott, 1965	
	Malagasya Cumberlidge & Sternberg, 2002	
	Marojejy Cumberlidge, Boyko & Harvey, 2000	
	Skelosophusa Ng & Takeda, 1994	
[1]Hydrothelphusinae Bott, 1955 (14)		*Afrithelphusa*
		Boreathelphusa
		[2] *Globonautes*
		Hydrothelphusa
		[2]*Louisea*
		Madagapotamon
		Malagasya
		Marojejy
		Skelosophusa

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CHAPTER 2

DISENTANGLING THE DIVERGENCE AND CLADOGENESIS IN THE FRESHWATER CRAB SPECIES (POTAMONAUTIDAE: *POTAMONAUTES PERLATUS* SENSU LATO) IN THE CAPE FOLD MOUNTAINS, SOUTH AFRICA, WITH THE DESCRIPTION OF TWO NOVEL CRYPTIC LINEAGES [*]

ABSTRACT

River networks of major drainages can form barriers that shape the phylogeography of freshwater organisms, particularly those with low dispersal capabilities. Freshwater crab species' distributions can be used to examine hydrological patterns to expose historical drainage interconnectivity. I used molecular sequence data (mitochondrial and nuclear DNA) and divergence time estimations to determine the phylogeography of the freshwater crab, *Potamonautes perlatus* sensu lato, from six drainage systems along the Cape Fold Mountains, South Africa. Two major clades were detected: clade 1 comprised two geographically discrete haploclades occurring in southern flowing drainages, while clade 2 included specimens from western flowing drainages. Divergence time estimations suggested a Pleistocene (*c.* 2.61 Mya) divergence of *P. perlatus* s.l. The Pleistocene was associated with arid conditions and drainage contractions. However, it is likely that during the mesic conditions of the Pleistocene, *P. perlatus* s.l. migrated and diverged into contemporary patterns. I conclude that three lineages are nested within *P. perlatus* s.l., two representing novel species. *Potamonautes perlatus* sensu stricto is confined to western flowing drainages. The two novel species both occurring in southern flowing drainages are described: *P. barbarai* sp. nov. occurs in the Gamtoos and Gourits Rivers and *P. barnardi* sp. nov. in the Breede River.

INTRODUCTION

Population genetic structure is sculpted by habitat discontinuity and habitat fragmentation, coupled with the dispersal capability of individuals within species, and major historical geologic and climatic events (MacArthur & Wilson, 1967; Flagstad *et al*., 2001; Hewitt, 2001; Emerson, 2002). Phylogeography can be used to improve the understanding of the mechanisms that influenced the spatial and temporal distribution of intra- and

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interspecific genetic variation (Avise *et al*., 1987; Hewitt, 2004; Avise, 2009). Freshwater habitats such as rivers provide the ideal template environments for exploring phylogeographic patterns of intraspecific population structure because most freshwater organisms are isolated to specific aquatic inland systems with either restricted or no dispersal between drainages (Avise & Felley, 1979; Gascon *et al*., 1998; Gascon *et al*., 2000; Hughes *et al*., 2004; Nicolas *et al*., 2011). Freshwater species with wide geographic distributions can therefore be used to examine hydrologic evolution (e.g. Avise & Felley, 1979; Nicolas *et al*., 2011).

The formation of hydrological networks is largely attributed to historical geologic and climatic events that resulted in changes in river flow patterns (Lowe-McConnell, 1987; Domínguez-Domínguez *et al*., 2006). Hydrological networks have been found to be effective barriers to gene flow for many terrestrial and amphibious vertebrates, birds and several freshwater species (Meffe & Vrijenhoek, 1988; Ward *et al*., 1994; Avise & Walker 1998; Avise *et al*., 1998; Gascon *et al*., 1998, 2000; Eriksson *et al*., 2004; Hughes *et al*., 2004; Domínguez-Domínguez *et al*., 2006; Hubert & Renno, 2006; Mock *et al*., 2010; Nicolas *et al*., 2011). Drainage evolution along the south-eastern and south-western coasts of South Africa, particularly drainage isolation, interconnection, as well as the development of hydrological basins, strongly reflects the history of the transformation of the Cape Fold Mountains (CFM) (Waters & Cambray, 1997). Two highly erosion-resistant belts of folded strata, which are parallel to the south coast and adjacent to the west coast (Boelhouwers & Meiklejohn, 2002), developed as a result of tectonic movements that occurred during the late-Permian and early Triassic 278 – 215 Mya (Lock, 1980; Cole, 1992; Deacon *et al*., 1992; McCarthy & Rubridge, 2005).

Major periods of cladogenesis of taxa along the CFM is estimated to have occurred between the Miocene and late Pleistocene, and the contemporary drainage patterns and distribution of many faunal groups were established by the late Pliocene (Deacon, 1983; Partridge & Maud, 1987; Linder, 2003; Daniels *et al*., 2006a; Waters & Cambray, 1997). The arrangement of the CFM mountains has been associated with breaks in the genetic structuring of freshwater vertebrates (e.g. Swartz *et al*., 2009) and terrestrial vertebrates (e.g. Swart *et al*., 2009; Daniels *et al*., 2007; Stanley *et al*., 2011), but there have only been limited studies on invertebrates. Freshwater invertebrates, such as freshwater crabs and crayfish are ideal animals for examining the influence of hydrological networks on genetic partitioning because these animals have direct development and complete their life cycle in freshwater, and are incapable of prolonged survival in sea water, rendering their evolutionary patterning closely

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linked with their freshwater habitat (Daniels, 2003; Daniels *et al*., 2006a, Klaus *et al*., 2009; Klaus *et al*., 2010, Toon *et al*., 2010).

A number of Afrotropical freshwater crab species have wide distribution ranges, which makes them ideal templates for examining phylogeographic patterns because they can provide information on historical drainage isolation. In South Africa, one of the most widely distributed freshwater crab species, *Potamonautes perlatus* H. Milne Edwards, 1837, has been the subject of two recent phylogeographic studies (Daniels, 2003; Daniels *et al*., 2006a). *Potamonautes perlatus* sensu lato occurs from the interior of the Northern Cape along the Cape Fold Mountains in the Western Cape into the Eastern Cape, with its range stretching over 900 km (Barnard, 1950; Daniels, 2003; Cumberlidge, 2013). Daniels (2003) used mitochondrial DNA (mtDNA) and allozyme electrophoresis to examine the genetic structure of *P. perlatus*, in the CFM within and between four major drainages, i.e. Berg, Breede, Gamtoos, and Olifants River systems. Although the allozyme results revealed genetic invariance between conspecific populations, the mtDNA results demonstrated that populations of *P. perlatus* s.l. comprised two distinct clades representing western flowing drainages (Berg sister to Olifants) and southern flowing drainages (Breede sister to Gamtoos), with the CFM serving as a potential barrier to gene flow (Daniels, 2003). These findings were corroborated by Daniels *et al*. (2006a) who sampled *P. perlatus* s.l. from an additional ten localities within the major river systems on the CFM. Daniels *et al*. (2006a) established that *P. perlatus* s.l. population structure in the CFM range may have been separated by tectonic activity (in the Miocene and Pliocene) to form segregated groups that subsequently underwent independent diversifications to form two monophyletic clades. The two clades correspond to the hydrological networks flowing in the different directions (south and west) (Daniels *et al*., 2006a). These conclusions were however dependent on a single locus and a general clock like assumption of the mtDNA locus used. No divergence time estimation was undertaken during the latter study.

The use of a single locus and, specifically, the presumed neutrality of mtDNA to obtain information on the hierarchical relatedness as well as the relative rates of evolution of species have been questioned (Bickford *et al*., 2007; Zink & Barrowclough, 2008; Edwards & Bensch, 2009). In particular, it has recently been argued that mtDNA can be unreliable for both taxonomic and phylogeographic differentiation for inter- and intraspecific genetic population structure because it only reflects the matrilineal evolutionary history which can differ from the overall genetic history of a specified population (Zhang & Hewitt, 2003; Ballard & Whitlock, 2004; Rubinoff & Holland, 2005; Edwards & Bensch, 2009; Mantooth

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& Riddle, 2011). Studies that rely on mtDNA can therefore result in erroneous inferences of population structure (Rubinoff & Holland, 2005; Song *et al*., 2008; Chu *et al*., 2009). Several authors have therefore suggested that the use of mtDNA in conjunction with multiple nuclear DNA (nuDNA) loci is a better approach to gain insight into evolutionary processes and patterns (Hudson & Coyne, 2002; Ballard & Whitlock, 2004; Bazin *et al*., 2006; Edwards *et al*., 2005; Elias *et al*., 2007; Koepfli *et al*., 2008; Chu *et al*., 2009). Because the evolution of nuDNA is slower compared to mtDNA, phylogenetic similarities between the two are expected to confirm boundaries between phylogroups (Rubinoff & Holland 2005; Brito & Edwards, 2009; del Cerro *et al*., 2010).

The objective of the present study was to perform phylogenetic analyses using a combination of mtDNA and nuDNA loci to examine the intraspecific variability among geographically isolated populations of *P. perlatus* s. 1. (common Cape river crab) occurring in the major drainages of the Cape Fold Mountain range. This was done to determine whether the same population genetic structuring observed by Daniels (2003) and Daniels *et al.* (2006a) can be inferred using multiple genetic markers. To date, studies relating to the phylogeography of *P. perlatus* s.l. on the CFM have been disjunct, relying on only allozyme data and focusing on single drainage systems, i.e. Olifants River (Daniels *et al*., 1999a) and Berg River (Daniels *et al*., 1999b). Furthermore, those studies relied on a small sample size (Daniels, 2003; Daniels *et al*., 2006a). In addition, the latter two studies demonstrated that two evolutionary units were potentially nested within *P. perlatus* s.l., however the absence of nuDNA sequence markers prevented the authors from making any firm conclusions regarding species boundaries. Therefore, I also describe two cryptic lineages nested within the *P. perlatus* s.l. species complex. Moreover, Daniels (2003) and Daniels *et al*. (2006a) estimated the divergence times of *P. perlatus* s.l. using outdated methods. In this study, I explore the phylogeography of *P. perlatus* s.l. through the addition of a nuDNA marker and the application of improved divergence time estimations for freshwater crab cladogenesis to infer the factors that may be causal to the current hydrology patterns. The southern flowing clade is divided into two lineages that are described as novel species in the present study.

MATERIALS AND METHODS

SAMPLE COLLECTION

Specimens of *Potamonautes perlatus* s.l. were collected from six major drainage systems in the Western Cape, South Africa (Daniels, 2003; Daniels *et al*., 2006a). These were

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the Gamtoos, Gourits, and Breede River drainages that all flow southward and the Berg, Olifants, and Eerste River drainages that all flow westward. Ox heart-baited lines were used to attract and capture the *P. perlatus* s.l. specimens. Crabs were killed by freezing them overnight at -20 °C, after which muscle tissue was extracted from the pereopods and stored in absolute ethanol for use in genetic analysis. A total of 122 specimens (1 to 5 per locality) of *P. perlatus* s.l. from 33 localities (tributaries) within the six drainages were used for analysis. These localities covered most of the large drainage systems in the species' known distribution range (Table 1; Fig. 1).

MOLECULAR TECHNIQUES AND SEQUENCING

DNA was extracted from the pereopod muscle (0.5 to 1 g of tissue) of each specimen (Daniels, 2003; Daniels *et al*., 2006a) using a QIAgen DNAeasy kit following the protocol outlined by the manufacturers. The extracted DNA was stored at -20 °C until required for polymerase chain reaction (PCR) (Daniels, 2003; Daniels *et al*., 2006a).

Before use in PCR, 1µL of the extracted DNA was diluted in 19 µL of millipore water. Two mitochondrial genetic markers (mtDNA) were used: cytochrome oxidase I (COI; LCOI-1490 5'-GGT CAA CAAA TCA TAAA GAT ATTG-3' and HCOI-2198 5'-TAAA CTT CAG GGT GAC CAAA AAA TCA-3') (Folmer *et al*., 1994) and 16S rRNA (16SA 5'-ACT TGA TAT ATA ATT AAA GGG CCG-3' and16SB 5'-CTG GCG CCG CTC TGA ACT CAA ATC-3') (Palumbi *et al*., 1991). One nuclear DNA (nuDNA) genetic marker, 28S rRNA (28Sa-modified, 5'-GAC CCG TCT TGA ARC ACG GA-3' and 28Sb, 5'-TCG GAA GGA ACC AGC TAC-3') (Jesse *et al*., 2010), was used in addition to the mtDNA. Using combined mtDNA and 28S rRNA, Jesse *et al*. (2010) detected the presence of a cryptic lineage within their data. Limited nuDNA sequence markers are currently available for low level phylogeographic analyses in freshwater crabs and no microsatellite library is available for any freshwater crab species. Most of the mtDNA (16S rRNA and COI) sequences were obtained from Daniels (2003) and Daniels *et al*. (2006a) under GenBank accession numbers AF493160 – AF493176, DQ028635 – DQ028734 for 16S rRNA, and AF494022 – AF510874 for COI. Additional COI sequences were obtained from the second author (unpublished). Moreover, because an unequal number of sequences were accessible for some localities, additional amplification was required for this study. Where amplification could not be achieved, the samples were omitted from further analyses (see Table 1 for the number of specimens used for COI and 16S rRNA per locality). For the 28S rRNA, only one specimen

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per locality was amplified. This is because the slower mutation rate of nuDNA (in this instance, 28S rRNA) compared to mtDNA (COI and 16S rRNA) makes it less variable (Ragionieri *et al*., 2009), and therefore, sequencing one specimen per locality was deemed sufficient to represent a sample locality. Missing specimens (i.e. those that could not be amplified for the 28S rRNA marker – totalling five specimens) were excluded from the analyses.

A 25 µL reaction solution for each sample was used for the PCR amplifications: millipore water (14.9 µL), 25 mM $MgCl_2$ (3.5 µL), 10X Mg^{2+} free buffer (2.5 µL), 10 mM dNTP solution (0.5 µL), 10 mM forward and reverse genetic marker primers (0.5 µL each), 0.1 U Taq polymerase (0.1 µL), 2.5 µL of the 1:19 template DNA dilution. For COI, the PCR conditions were 94°C (4 min.), [94°C (30 sec.), 42°C (40 sec.), 72°C (45 sec.)] for 36 cycles, and a final extension at 72°C (10 min.). The 16S rRNA was amplified under the following PCR conditions: at 95°C (5 min.), [95°C (30 sec), 50°C (40 sec.), 72°C (1 min.)] for 36 cycles, and 72°C (10 min.) final extension. The 28S rRNA PCR amplifications were set to 94°C (4 min.), [94°C (45 sec.), 50°C (1 min.), and 72°C (1 min.)] for 40 cycles, followed by a final extension at 72°C (10 min.).

The PCR products were electrophoresed for 4 hours in a 1% agarose gel containing ethidium bromide. The DNA fragments were then purified using the BioFlux purification kit (Bioer Technology Co., Ltd). The purified PCR products were sent to Macrogen Inc. (Seoul, South Korea; http://www.macrogen.com) for sequencing. Sequences were checked for ambiguities and aligned with Clustal W Multiple Alignment as executed in MEGA5 v. 2.2 (Tamura *et al*., 2011). Further adjustments to the sequences were done manually where obvious mismatches were made by the computational alignment. The COI sequences were translated to amino acids to check for stop codons using the online program EMBOSS-Transeq (http://www.ebi.ac.uk/emboss/transeq/); no stop codons were detected.

PHYLOGENETIC ANALYSES

Datasets from 16S rRNA and COI are linked in the mitochondria and are both maternally inherited and were therefore combined for analyses in the present study. Phylogenetic trees were rooted using *P. warreni* and *P. unispinus* as outgroups (Daniels *et al*., 2002; 2006a). Daniels *et al*. (2002) demonstrated that these two species formed a well-supported monophyletic group with other large-bodied freshwater crab species, including *P. perlatus* s.l.

Maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) were used to construct phylogenetic trees. The MP and ML analyses were executed in MEGA5 v. 2.2 (Tamura *et al*., 2011). For the MP analyses, the evolutionary histories were estimated by constructing a bootstrap consensus tree inferred from 1000 replicates taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates), are shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Close-Neighbour-Interchange algorithm (Nei & Kumar, 2000) and the initial trees were obtained with the random addition of sequences (ten replicates). The tree was drawn to scale with branch lengths calculated using the average pathway method (Nei & Kumar, 2000) and are in the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated.

The ML bootstrap trees were inferred from 10 10^3 replicates that were taken to represent the evolutionary history of the taxa analyzed. The Tamura 3-parameter model with gamma-distributed rate heterogeneity (T92 + G) obtained in MEGA5 v. 2.2 (Nei & Kumar, 2000; Tamura *et al*., 2011) was used for analysis. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated.

For the BI analyses, the best-fit models for the mtDNA as well as the nuDNA combined with mtDNA (1575 bp) datasets were obtained by using jModelTest v. 2.1.3 (Posada, 2008). The best-fit maximum likelihood scores were chosen using Akaike's Information Criterion (AIC) (Akaike, 1973) in order to separate more complex models, and reduce the amount of unnecessary parameters that contribute little to describing the data (Burnham & Anderson, 2002; Nylander *et al*., 2004). The models were used for phylogenetic reconstruction using BI in MrBayes v. 3.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003; Ronquist *et al*., 2012). BI consensus trees were constructed in a partitioned analysis using a substitution model for each gene. Four Markov Chain Monte Carlo (MCMC) simulations were run for 5×10^6 generations with each chain starting from a random tree and sampling from the chain was conducted every 5×10^3 generations. Convergence was reached when the standard deviation of split frequencies was < 0.01 (Ronquist & Huelsenbeck, 2003; Ronquist *et al*., 2012). A 50% majority rule tree was retained after a 25% burn-in. Consensus trees were viewed and edited in FigTree v. 1.4

(Drummond *et al*. 2009). Branches with posterior probabilities (p*P*) ≥0.95 were regarded as well supported.

POPULATION STRUCTURE

Estimates of evolutionary divergence (uncorrected "p" distance) over sequence pairs between the major clades recovered from the phylogenetic reconstructions were conducted in MEGA5 v. 2.2 (Tamura *et al*., 2011). All positions containing gaps and missing data were eliminated, leaving a total of 601 positions in the final dataset. Population structure was investigated using the COI gene locus because it was the most variable mitochondrial gene. TCS v. 1.21 (Templeton & Sing, 1992; Crandall & Templeton, 1996; Clement, Posada & Crandall, 2001) was used to construct two independent 95% parsimony haplotype network for the COI and 28S rRNA datasets, the fastest and slowest evolving genetic markers, respectively.

To estimate population genetic differentiation across all localities (Φ_{ST}), an analysis of molecular variance (AMOVA) was conducted for the COI gene in Arlequin v. 3.5.1.2 (Excoffier *et al*., 2005). AMOVA was also performed for the two large haplogroups and Φ_{ST} values were calculated between sample localities to determine whether populations differed significantly in their genetic composition. Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997) were used to examine selective neutrality among haplotypes. Negative values for both Tajima's *D* and Fu's F_S are expected under population expansion (Tajima, 1989; Fu, 1997), while positive values for Tajima's *D* would be indicative of population decline.

DIVERGENCE TIME ESTIMATIONS

Divergence times for the combined mt- and nuDNA (COI, 16S rRNA, and 28S rRNA) dataset were estimated in BEAST v.2.0.2 (Drummond *et al*., 2002, 2009, 2012a, b). Only mtDNA trees were linked for analysis because they are linked in the mitochondria. Even though it has been has been suggested that phylogenetic dating using shallow phylogenies and slow evolving or less variable genetic markers has minimal influence on the posterior (Brown & Yang, 2010), I still included the nuDNA marker under a broad uniform substitution rate prior as there is no generally accepted mutation rate for this marker. Fossil records for African freshwater crabs are limited, and no fossil species have been described from South Africa (Daniels *et al*., 2006b; Daniels, 2011). In addition, there is no single vicariance event that can be used for dating the Afrotropical freshwater crab clades (Daniels

et al., 2006b; Klaus *et al*., 2011). Therefore, mutation rates for the two mtDNA markers (16S rRNA and COI) were used to estimate divergence times (Jesse *et al*., 2010; Daniels, 2011; Shih *et al*., 2011; Klaus *et al*., 2013). For COI, a mean mutation rate of 2.0 x 10^{-8} (with a range of 6.40 x 10^{-9} – 1.42 x 10^{-8} and a standard deviation (SD) of 3.059 x10^{-9}) was used. A mean mutation rate of 1.02 x 10^{-8} (with a range of 1.40 x 10^{-8} – 2.60 x 10^{-8}; SD = 1.94 x 10^{-9}) per Myr was used for 16S rRNA. Both of these rates 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). The 28S rRNA rate was estimated around the mutation rates of the two mtDNA markers.

The BEAST input (xml) file was created in BEAUti v.2.0.2 (Drummond & Rambaut, 2007; Drummond *et al*., 2012a, b). After a series of preliminary test runs, the Yule speciation process (Heled & Drummond, 2012) 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. The substitution models as obtained from jModelTest v. 2.1.3 and clock rates were unlinked for all genes, while the mtDNA trees were linked and the nuDNA tree was unlinked from the mtDNA tree. Four MCMC chains were run four times for 200 x 10^6 iterations, chains and trees were sampled every 20 x 10^3 generations. The convergence of the four combined chains (in LogCombiner v.2.0.2, part of the BEAST software package) was verified by an effective sample size (ESS) greater than 200 for each parameter in Tracer v. 1.5 (Drummond & Rambaut, 2007; Drummond *et al*., 2009). The resulting trees from the four chains were combined and a maximum clade credibility tree was computed with mean heights in TreeAnnotator v. 2.0.2 after a 10% burn-in was discarded. FigTree v. 1.4 was used to visualize the tree (Drummond *et al*., 2009).

MORPHOLOGY

The tree topology retrieved three lineages within *P. perlatus* s.l. One of these lineages is designated as *P. perlatus sensu stricto*, while the two remaining lineages are described. Diagnostic morphological characters (i.e. the carapace, chelipeds, gonopods 1 and 2, mandibular palp, and the sternum) were examined between the three lineages using a Leica EC 3X stereomicroscope. Images of gonopods 1 and 2 for each of the three were captured with a Leica MZ 16A digital camera attached to a Leica EC 3X stereomicroscope. An extensive analysis of specimens in the collection of the South African Museum of Natural

History (SAM), Iziko Museums of Cape Town was undertaken and specimens were assigned to new lineages based on geographic concordance.

RESULTS

PHYLOGENETIC RESULTS

Combined mtDNA (COI and 16S rRNA) tree topology

A total of 628 bp were obtained for COI, while the 16S rRNA sequence data yielded 370 bp (n = 122 for each mtDNA locus). The combined mtDNA sequence data set (COI and 16S rRNA) yielded a total of 998 bp. For the COI locus, TPM1uf + G was selected as the best-fit the model, while the TPM3uf + I + G model was selected for the 16S rRNA locus (see Table 2 for detailed information for each model). Generally, all three analytical methods revealed two statistically well supported clades comprising freshwater crabs occurring in the southern- and western flowing drainages (Fig. 2). Clade 1 comprised the southern flowing drainages (Breede, Gamtoos, and Gourits), which was further divided into two well-supported clades (1A and 1B). Clade 1A comprised an admixture of populations from the Gamtoos and Gourits drainage systems (ML = 76%, MP = 99%, *Pp* = 0.98). Clade 1B comprised freshwater crabs from the Breede River drainage system (ML < 75%, MP < 75%, p*P* < 0.95). Clade 2 consisted of freshwater crabs from the western flowing drainage systems (Berg, Eerste, and Olifants Rivers). While this group was poorly supported by ML, both MP and BI showed strong statistical support (97% and 0.97 p*P*, respectively). This group was also subdivided into two statistically supported clades, the Olifants and Berg River group (ML and MP < 70%, p*P* = 0.96) as well as the Eerste River freshwater crabs, which grouped with the Tokai and Liesbeeck (ML < 70%, MP = 93%, p*P* = 0.97).

Combined mtDNA and nuDNA tree topology

The 28S rRNA locus yielded 577 bp and this locus best-fit the TVM + G model (Table 2). The combined mtDNA and nuDNA yielded 1575 bp. With the exception of the Tunnel Terminals specimens, the combined mtDNA and nuDNA topology (Fig. 3) was congruent with the mtDNA phylogenetic reconstructions. The phylogenetic reconstruction of the combined mtDNA and nuDNA yielded two well-supported clades. Clade 1 comprised freshwater crabs from the Gamtoos, Gourits, and Breede River, which are the southern flowing drainage systems. As with the mtDNA phylogenetic reconstructions, clade 1 was

divided into two clades (1A and 1B). Clade 1A comprised an admixture of specimens from the Gamtoos and Gourits drainages systems (ML = 79%, MP < 75%, p*P* = 1.00). Tunnel Terminals, which previously grouped within the Breede River freshwater crabs (Fig. 2), now grouped with the Gamtoos / Gourits drainage systems group in clade 1A and clade 1B comprised specimens from the Breede River system (ML = 81%, MP = 93%, p*P* = 1.00). Unlike the mtDNA phylogenetic reconstruction, the addition of 28S rRNA resulted in the specimens from Breede River becoming one group. Similarly, the populations from the western flowing drainages clustered together in one group as opposed to the two groups obtained from the mtDNA analysis. Clade 2 (Berg, Eerste, and Olifants drainage systems) was statistically supported by MP and BI (75% and 1.00 p*P*, respectively), but was not supported by ML.

POPULATION STRUCTURE

The mean evolutionary divergence between sequence pairs of the major clades recovered from phylogenetic reconstructions revealed a 10.7% (min = 7.3%, max = 20.3%) sequences divergence between clade 1A and 1B, 10.7% (min 7.3%, max = 13.3%) between clade 1A and clade 2, and 4.8% (min = 3.8%, max 5.0%) between clade 1B and clade 2. TCS collapsed the 122 COI sequences into 34 haplotypes, with haplotype frequencies ranging from 1 to 29 (Fig. 4) and no haplotypes shared between the two clades. The COI haplotype network corroborated the phylogenetic tree constructed for both the mt- and nuDNA (Figs. 2 and 3), and retrieved two major haploclades comprising the southern- and western flowing drainage systems. Haplotypes from the Breede River drainage system (Bainskloof, Bonnievale, De Hoop, Klein River, Robertson, and Tunnel Terminals) could not be connected to the Gamtoos / Gourits group with 95% confidence by TCS. The nuDNA (28S rRNA) haplotype network (25 haplotypes) showed some shared haplotypes between drainage systems (Fig. 5), which is expected with slower evolving genes.

Over all localities, the AMOVA results revealed that 87.26% of the total variance was explained by the variation among localities (df = 32, Va = 12.86), while 12.74% (df = 89, Vb =1.88) was explained by the variation within localities (Table 3). The genetic structure between the two clades showed that the variation in the southern flowing drainage systems (clade 1, Figs. 2 and 4) was lower (63.59% df = 16, Va = 0.67) than that of the western flowing drainage systems (95.07% df = 9, Va = 3.94) (clade 2, Figs. 2 and 4). Within clade 1, the variation within populations was explained by 36.41% (df = 57, Vb = 0.39), while the

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variation within clade 2's the populations was explained by 4.93% (df = 21, Vb = 0.21). AMOVA indicated significant population structure (overall localities: Φ_{ST} = 0.85; clade 1 (southern flowing drainages): Φ_{ST} = 0.64; and clade 2 (western flowing drainages): Φ_{ST} = 0.95]. Both Tajima's *D* and Fu's F_S were positive for overall populations, which is consistent with a decrease in population size (Tajima, 1989; Fu, 1997). In contrast, Tajima's *D* was negative for clades 1 and 2, suggesting that population expansion had occurred within *P. perlatus* s.l. occurring in both the southern- and western flowing drainage systems.

DIVERGENCE TIME ESTIMATIONS

The divergence of *P. perlatus* s.l. occurred during the early Pleistocene, between 2.61 Mya (95% highest posterior density (HPD): 0.96 – 5.07 Mya) and 0.08 Mya (95% HPD: 0.21 – 1.68 Mya) (Fig. 6). The Olifants River (western flowing; clade 2, Figs. 2 and 3) population diverged from *P. perlatus* s.l. over 2.61 Mya, while the Gamtoos / Gourits populations diverged from the Breede River populations 1.40 Mya (95% HPD: 0.40 – 3.3 Mya). The admixture of populations from the Gamtoos and Gourits River populations that was observed from the phylogenetic reconstructions was also observed in the divergence time tree obtained from the BEAST analyses. Within the Gamtoos / Gourits clade (clade 1a, Figs. 2 and 3) divergence occurred from about 0.08 Mya (95% HPD: 0.21 – 1.68 Mya), while within the Breede River (clade 1b; Figs. 2 and 3), divergence occurred 0.56 Mya (95% HPD: 0.09 – 1.30 Mya). Divergence within the Olifants River (clade 2, Figs. 2 and 3) occurred from 0.94 Mya (95% HPD: 0.20 – 2.07 Mya).

MORPHOLOGY

The examination of the carapace, chelipeds, gonopods 1 and 2, mandibular palp, and the sternum revealed no diagnostic differences between the three clades, hence the genetic data was used to delineate the novel lineages.

DISCUSSION

Two major geographically discrete clades (1 and 2, Fig. 2 and Fig. 3) were present throughout the distribution of *P. perlatus* s.l. and corroborated the earlier results by Daniels (2003) and Daniels *et al*. (2006a), with clade 1 comprising two sub-clades (A and B). However, while previous research concluded that the divergence between these two clades occurred during the Miocene (Daniels *et al*., 2006a) and Pliocene (Daniels, 2003), this study

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estimated that divergence occurred much more recently in the Pleistocene between 2.61 (95% HPD: 0.96 – 5.07 Mya) and 0.08 Mya (95% HPD: 0.21 – 1.68 Mya). However, these divergence times should be interpreted with caution, particularly because of the high 95% HPD range, which reflect the high level of uncertainty of this divergence time estimation method. Although the mean (point) divergence times are given by the programme, divergence may have occurred at any period within the 95% HPD range, as far back as the Pliocene. Moreover, while the mutation rate of the nuDNA marker can be estimated from the two mtDNA markers, the divergence time may be over- or underestimated because the trees from the two genomes cannot be linked.

The population genetics data suggest that the *P. perlatus* s.l. populations have low or limited dispersal between localities (as demonstrated by the high $\Phi_{ST} = 0.85$) for all localities in the southern- and western flowing drainage systems. These findings were further substantiated by the neutrality tests, which demonstrated that populations have undergone a past population contraction (or a genetic bottleneck) or subdivision as indicated by the positive Tajima's D and Fu's F_S (Tajima, 1989; Fu, 1997). When considering the two clades separately, I found that freshwater crabs occurring in southern flowing drainages (clade 1) were weakly, but significantly, structured ($\Phi_{ST} = 0.64$), and this was supported by the phylogenetic reconstruction of clade 1A which showed an admixture of the Gamtoos and Gourits river populations, with no clear genetic breaks between localities. Clade 2, comprising the western flowing drainages (Berg and Olifants), displayed the highest Φ_{ST} value (0.95, Table 3), which, like the overall localities data, is suggestive of low dispersal and limited genetic admixture between sampled localities. However, the neutrality tests (Tajima's D and Fu's F_S) for the two clades were not statistically significant, limiting any further inferences.

The phylogenetic reconstructions revealed two well-supported clades within *P. perlatus* s.l. The lack of support by the mtDNA phylogenetic reconstruction for the inclusion of the Breede River clade corresponded with the findings of the parsimony haplotype network. However, as hypothesized by Daniels *et al.* (2006a), the nuclear marker corroborated a historical connection within and between the two major clades of the southern flowing drainages. Indeed, the combined nuDNA and mtDNA phylogenetic reconstructions revealed that the Tunnel Terminals population was historically genetically similar to the Gamtoos/Gourits group, as shown by the addition of the slower evolving nuDNA genetic marker (Fig. 3). This suggests two possible historical drainage connections. First, the Tunnel Terminals *P. perlatus* s.l. specimens occur in a tributary of the Berg River, however,

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phylogenetics show that this population genetically grouped with Breede River populations. Second, the presence of Tunnel Terminals within the Gamtoos / Gourits drainage systems also suggests an historical drainage connection between these southern flowing drainages and the Berg River (western flowing drainage), as well as the Breede river.

In their study of redfin minnows, *Pseudobarbus burchelli*, Swartz *et al*. (2009) concluded that the Gourits River drainage system was an important link between the southern flowing drainages in the diversification of redfin minnows, providing inland connection for their dispersal during wetter periods in the Pleistocene. Chakona *et al*. (2013) also suggested a similar dispersal mechanism for *Galaxias* sp. 'nebula' during the Pleistocene and Holocene. Moreover, Swartz *et al*. (2009) hypothesized that the isolation of the Breede River populations of *Ps. burchelli* was due to those populations being unable to spread to the current Gourits River drainage system after the Last Glacial Maximum. However, while the divergence time results of the current study revealed that the Breede River freshwater crab populations have been isolated from the Gamtoos/Gourits populations for over 1.8 Myr, Swartz *et al*. (2009) suggested that, according to their findings with minnows, the current Gamtoos / Gourits and Breede river captures were no more than 18 000 years old. Nonetheless, for *P. perlatus* s.l., divergence time based on mtDNA sequences suggests that these populations may not have had contact for approximately 1.8 Myr, which is inconsistent with Swartz *et al*.'s (2009) findings.

The divergence time cladogram revealed a divergence spanning the last 2.4 Myr, with the ancestral lineages of *P. perlatus* s.l. experiencing significant dispersal and population expansion during the Pleistocene. The divergence of *P. perlatus* s.l. populations was generally found in this study to be associated with events in the Pleistocene, which contradicts Daniels (2003) and Daniels *et al*.'s (2006a) conclusions, where divergence was estimated to have occurred in the Pliocene and Miocene, respectively. However, in another study, Daniels *et al*. (2001) estimated the divergence of freshwater crabs occurring in the Cape Peninsula to have occurred between the late Miocene / Pliocene and between the late Pliocene / Pleistocene. Daniels *et al*. (2001) attributed their findings to climatological and geological changes that occurred from the Miocene onwards, which resulted in the contraction and expansion of inland taxa. These divergence time estimations (Daniels *et al*., 2001; Daniels, 2003; Daniels *et al*., 2006a) are however plausible because the drainage systems of the CFM were subject to high levels of precipitation during the Miocene, while arid conditions were prevalent during the Pliocene and Pleistocene (Cowling *et al*., 2008). The late Pliocene and early Pleistocene (from *c*. 3 Mya to 1.6 Mya) have been associated with

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wet/ dry cycles, which are said to have peaked between 1.8 Mya and 1.6 Mya (Chase & Meadows, 2007; Linder, 2005). I hypothesize that these cycles may have been responsible for the divergence of *P. perlatus* s.l. While freshwater crabs are known to have low dispersal capabilities (Daniels, pers. comm.), the limited studies that have been published suggested that freshwater crabs are capable of short distance dispersal over land in highly humid conditions (Gherardi *et al*., 1988a; Gherardi *et al*., 1988b). Gherardi *et al*. (1988a) reported that females migrated further from streams to the surrounding terrestrial habitat than males. Therefore, *P. perlatus* s.l. may have taken advantage of the wet cycles of the Pleistocene by migrating to neighbouring streams. Ultimately, the shrinkage or complete disappearance of some smaller river systems may have limited the re-expansion of *P. perlatus* s.l. populations during xeric conditions.

Previous studies support the theory that historic geological and climatic events associated with the late Miocene to the Pleistocene on the CFM coincided with the drastic transformation of the population histories of terrestrial flora and inland aquatic fauna (Cowling *et al*., 2008; Swartz *et al*., 2009). In spite of this, a growing number of studies involving other taxa suggest that divergence may have occurred during the Pleistocene (Tolley *et al*., 2006; Daniels *et al*., 2007; Price *et al*., 2007; Swart *et al*., 2009). For example, a study investigating the divergence of terrestrial invertebrates (cicadas, *Platypleura stridula*), that are host-plant specific and occur on plants found near drainages on the CFM, showed that the large genetic distances between lineages were a result of the isolation of drainages, which may have reduced gene flow between drainages during the Pleistocene (Price *et al*., 2007). Price *et al*. (2007) attributed their findings of the diversification of cicadas in the Pleistocene to possible habitat philopatry. In addition, several other animal studies have shown that the Cape Fold Mountains are a major barrier for gene flow (Tolley *et al*., 2006; Daniels *et al*., 2007; Swart *et al*., 2009). For reptiles (lizards and chameleons), which are habitat specific, it was found that Pliocene and Pleistocene climatic conditions also greatly influenced their genetic structure between western and eastern clades occurring on the CFM (Tolley *et al*., 2006; Swart *et al*., 2009). In another example, Daniels *et al*. (2007) found that ungulate tortoise populations were divided into south-western and southern populations as a result of the barriers caused by the folding of the CFM. Although Daniels *et al*. (2007) ascribed this genetic structuring to the Miocene, the split between populations is consistent with the findings of this present study, where *P. perlatus* s.l. populations were genetically separated into western- and southern flowing drainage systems.

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With reference to the relationship between the southern- and western flowing drainages, the divergence time estimations revealed a pattern that is worthy of note. The divergence time estimates suggest that the Olifants River drainage populations were first to diverge from the most recent common ancestor. According to minimum spanning network distances (not shown), the western flowing drainages populations exhibited 21 mutational steps or extinct haplotypes from the southern flowing drainages populations. There were 31 mutational steps or extinct haplotypes between the Gamtoos / Gourits populations and Breede River populations. The latter two findings and the minimum uncorrected sequence divergence are a clear indication of cryptic speciation. Moreover, the inclusion of the nuDNA marker supported the monophyly of the *P. perlatus* s.l. (clade 2). I therefore propose that there are three lineages, two cryptic, within the currently known *P. perlatus* s.l. species complex. Therefore, a morphological re-examination of the CFM's *P. perlatus* s.l. populations is necessary in order to corroborate this.

Nevertheless, besides studies on freshwater crabs, there is a considerable paucity of comparative research examining the influence of major drainage systems as well as past geologic events on the genetic structure of invertebrate freshwater taxa occurring in the CFM, and in South Africa's drainage systems. In the present study I recognize the southern flowing drainage clade as two novel cryptic species (clade 1A and clade 1B, Fig. 2), based on the sequence divergence, geographic exclusivity and marked genetic differentiation. These two cryptic lineages are described in the present study.

SYSTEMATICS

SUBORDER BRACHYURA LINNAEUS, 1758

SUPERFAMILY POTAMOIDEA ORTMANN, 1896

FAMILY POTAMONAUTIDAE BOTT, 1970

SUBFAMILY POTAMONAUTINAE BOTT, 1970

GENUS *POTAMONAUTES* MAC LEAY, 1838

Potamonautes perlatus H. Milne Edwards, 1837

Thelphusa perlata H. Milne Edwards, 1837: 13

Thelphusa (Potamonautes) perlata (Milne Edwards) Mac Leay, 1838: 64

Thelphusa perlata (Milne Edwards) Krauss, 1843: 37

Thelphusa perlata White, 1847: 30

Thelphusa perlata H. Milne Edwards, 1853: 209

Thelphusa perlata (M. Edwards) Stimpson, 1858: 101

Thelphusa perlata (Milne-Edwards) Heller, 1865: 31

Thelphusa cristata A. Milne-Edwards, 1869: 180, plate 11 Fig. 1

Thelphusa perlata H. Milne Edwards, 1869: 179, plate. 9 fig. 3, 3a

Thelphusa perlata (A. Edwards) Ozorio, 1884: 226

Thelphusa (Potamonautes) perlata (Milne-Edwards) Miers, 1886: 215

Thelphusa perlata A. Milne-Edwards, 1887, 129

Thelphusa (Potamonautes) perlata (Milne-Edwards) Ortmann, 1893: 489

Thelphusa perlata (M. Edwards) Webber, 1897: 156,178

Potamon (Potamonautes) perlatum (Milne-Edwards) Ortmann, 1897: 304,307

Potamon (Potamonautes) cristatum (A. Milne-Edwards) Ortmann, 1897: 304,307

Potamon (Potamonautes) perlatus (Milne-Edwards) Rathbun, 1900: 284

Potamon (Potamonautes) perlatum (Milne-Edwards) Doflein, 1904: 105

Potamon (Potamonautes) perlatus Rathbun, 1904: plate. 14 Fig. 4

Potamon (Potamonautes) perlatus (Milne-Edwards) Rathbun, 1905: 163

Thelphusa perlata (Milne-Edwards) Ozorio, 1905: 149

Potamonautes perlatus (Milne-Edwards) Stebbing, 1905: 33

Thelphusa perlata (Milne-Edwards) Stimpson, 1907:113

Potamon (Potamonautes) perlatus (Milne-Edwards) Stimpson, 1907: 113, footnote

Potamonautes perlatus (Milne-Edwards) Stebbing, 1910: 293

Potamon (Potamonautes) perlatus (Milne-Edwards) Lenz, 1910b: 5 (124)

Potamon (Potamonautes) perlatus Rathbun, 1921: 417

Potamonautes perlatus (Milne-Edwards) Balss, 1922: 71

Potamon (Potamonautes) perlatus (Milne-Edwards) Colosi, 1924: 2-4, fig. 1

Potamon (Potamonautes) perlatus (Milne-Edwards) Barnard, 1929: 62

Potamonautes perlatus (Milne-Edwards) Barnard, 1935: 482, figs 1a and b

Potamonautes perlatus (H. Milne Edwards) Balss, 1936: 184, fig. 20

Potamon perlatus (H. Milne Edwards) Chace, 1942:219; Capart, 1954: 842, Fig. 18, 38

Potamon (*Potamonautes*) *perlatus* Barnard, 1950: 183-187, figs. 34a, 35 a-c

Potamonautes (*Potamonautes*) *perlatus perlatus* Bott, 1955: 254, plate 11, figs. 2a-c, 3, fig. 26

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Potamonautes perlatus sensu stricto (Clade 2, Fig. 2)

Material examined

Holotype / neotype: The holotype is dry and could not be sent for comparison. However, due to the uncertainty of the geographic provenance of the holotype, I designated a specimen from the western flowing drainages (housed at the SAM, Iziko Museums of Cape Town) as a neotype. The neotype was designated because the holotype from Milne-Edwards 1937 housed at the Museum of Natural History (catalogued: B4360), which, to my knowledge, was the first documentation of *P. perlatus*. The museum would not send the dried specimen for morphological comparison and I was therefore unable to conduct any DNA analyses. Moreover, the specimen was collected from the Cape Province at the Cape of Good Hope in South Africa. Though arbitrary, because as with many specimens collected before the use of geographical positioning systems, precise collection records are not available. Cape of Good Hope is situated on the west coast of South Africa, and I believe that the specimen could have been collected from any of the western flowing rivers that are close to the Peninsula. Historically, this species was thought to have a wide geographic distribution ranging from the Democratic Republic of the Congo (formerly Zaire), into Namibia (formerly South West Africa), and South Africa. However, there is no genetic evidence to prove this to be true. According to the revised taxonomy of the Potamonautidae (Chapter 6, this thesis), *P. perlatus* s.s. is a South African freshwater crab species and does not share close phylogenetic relationships with any of the Central or West African freshwater crab taxa. Moreover, divergence time estimations (Chapter 6) indicate that the clade that comprises *P. perlatus* s.s. (and the two cryptic lineages described here) diverged from its closest relatives occurring in Angola approximately 24.73 Mya (95% HPD: 12.55 – 47.27 Mya), making it unlikely that the specimens collected from Central and West Africa are *P. perlatus*. The precise geographic provenance of the holotype (deposited in the Museum of Natural History, Paris, Accession: B 4360) is unknown. However, it is clear that the name *P. perlatus* was historically applied to a number of distinct poorly defined species. In the present study, following the genetic evidence, I restrict the use of the name of *P. perlatus* to all the specimens in the western flowing drainages of the Western Cape Province of South Africa (clade 2, Fig. 2). This designation is done to stabilize the taxonomy of the species complex in line with the guidelines set by the International Code of Zoological Nomenclature (ICZN), Article 75 (75.3.1: "... to clarify the taxonomic status or the type locality of a nominal taxon"

and 75.3.5: “evidence that the neotype came as nearly as practicable from the original type locality and, where relevant, from the same geological horizon ... as the original name-bearing type” (http://www.nhm.ac.uk/hosted-sites/iczn/code/index.jsp?article=75&nfv=true).

Neotype: Krieddouwkrans, SAM A45755 (1 ♂), 32°21’59.76”S; 18°57’0’E, collected on 10.09.1994 by M. Somers. Morphological measurements of the neotype: carapace length (CL) = 41.4 mm; carapace width at the widest point (CWW) = 60.5 mm, carapace width at posterior margin (CWP) = 19.0 mm, frontal width (FW) = 41.0 mm, distance between postfrontal crest and anterior margin (PFCD) = 5.3 mm, carapace height (CH) = 28.4 mm, major cheliped propodus length (MCPL) = 55.0 mm, major cheliped propodus height (MCPH) = 23.8 mm, pereopod 2 merus length (P2ML) = 24.3 mm, pereopod 2 merus width (P2MW) = 8.8 mm, pereopod 5 merus length (P5ML) = 22.2 mm, and pereopod 5 merus width (P5MW) = 7.7 mm.

Additional material examined: Jonkershoek (Stellenbosch, Eerste River drainage system), SAM A41142 (1 ♂, 1 ♀), 33°57’60”S; 18°57’60”E, collected on 25.03.1994 by M. van der Merwe and L. Hoenson; Jonkershoek (Stellenbosch, Lang River, Eerste River drainage system), SAM A41215 (5 ♂, 1 ♀), 33°57’60”S; 18°57’60”E, collected on 17.11.1994 by J. Hulley; Tokai (Cape Peninsula), SAM A41137 (3 ♂, 9 ♀), 34°03’60”S; 18°25’60”E, collected on 29.05.1992 by B. Stewart and L. Hoenson; and Paarl Rock (Paarl, Berg River drainage system), SAM A41306 (1 ♂, 1 ♀), 31°43’60”S; 18°55’60”E, collected on 05.09.1994 by J. Hulley and L. Hoenson. The following are all from the Olifants River drainage system: Boontjieskloof (Cedarberg), SAM A44980 (1 ♀), no GPS coordinate, collected on 14.04.1996 by S.R. Daniels and J. Leaner, Cederberg (Cape), SAM A41166 (2 ♀), 32°25’60”S; 19°04’60”E, collected on 12.12.1972, no collector’s name; Doringbos (near Clanwilliam), SAM A41191 (1 ♂), 33°14’59.64”S; 19°19’60”E, collected on 20.02.1970 by B.F. Kensley; Keurbos (Rondegat River), SAM A41305 (1 ♀) and SAM A41186 (1 ♂, 2 ♀), no GPS coordinate, collected on 08.12.1994 and 10.09.1994 (respectively) by M. Somers; Krieddouwkrans, SAM A41187 (5 ♂), 32°21’60”S; 18°57’00”E, collected on 10.09.1994 by M. Somers; Olifants River (above Clanwilliam Dam, Olifants River drainage system), SAM A41216 (2 ♂, 2 ♀), 32°12’60”S; 18°54’60”E, collected on 25.11.1994 by M. Somers.

Description and diagnosis: The carapace margin is smooth ending in a reduced epibrachial tooth at the anterolateral margins of the carapace. The species has a carapace depth (CH/CL) of 0.69 mm and a posterior margin width (CWP/CL) of 0.46 mm. The post frontal crest is complete with a groove in the middle. The sternites, maxillipeds and mandibular palp are characteristic of *Potamonautes* (Cumberlidge & Tavares, 2006). Males

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are heterochelous with the right cheliped being larger than the left and the dactylus of the right cheliped is slightly arched. Females have equal size chelipeds. The cheliped teeth have sharp unequal serrations. The pereopods are long and broad; pereopod two is the longest. The abdomen is triangular and widest at segment 3 and narrowest at the telson. The posterior view of the terminal segment of gonopod 1 points away from the midline and its terminal point curves left and slightly upward (Fig. 7A) and the margins of the subterminal of gonopod 1 have numerous setae (Figs. 7A and 7B). The terminal segment of gonopod 2 (posterior view) is slanted away from the midline (Fig. 7C). The subterminal segment of gonopod 2 is approximately two thirds the length of gonopod 1.

Distribution: *Potamonautes perlatus* s. s is restricted to western flowing drainages in the Western Cape, South Africa, occurring from the Olifants River in the Cederberg Mountains, to the Cape Peninsula, Eerste River, and Berg River. Based on the current data, it is not clear whether this species occurs in sympatry with *P. barbarai* sp. nov., which also occurs in the Berg River drainage system in LaMotte Forest. In the Olifants River, *P. perlatus* is restricted to all the localities above Bulshoek dam. *Potamonautes granularis* Daniels, Stewart, & Coke 1998 occurs below the Bulshoek dam wall where the two species occur in sympatry.

Remarks: There are no visible differences in the diagnostic morphological characters between this species and the two novel lineages described below. In particular, no differences were found between the gonopods of the three lineages (Fig. 7 (A-I).

Etymology: Potamonautes perlatus s.s. was previously regarded as a widespread species. With the use of genetic evidence, I restrict the name to specimens of *P. perlatus* occurring in western flowing drainages.

Conservation: The conservation status of *P. perlatus* s.s. is Least Concern (Cumberlidge, 2013). However, the distribution of the species (*P. perlatus* s.s.) has now been narrowed with the description of two novel lineages within the species complex. According to Cumberlidge (2013), current and future threats to *P. perlatus* s.l. in its presumed distribution range are habitat loss and degradation. Therefore, I consider the species to be Data Deficient.

POTAMONAUTES BARBARAI SP. NOV. (CLADE 1A, FIG. 2)

MATERIAL EXAMINED

Holotype: Groot River (Gourits drainage system), 33°43'36"S; 24°37'14"E, SAM A41061 (1 ♂), collected on 09.11.1984 by D. Coetzee. This haplotype is endemic to the Gamtoos and Gourits drainage systems. Morphological measurements of the holotype: CL = 43.2 mm; CWW = 63.0 mm, CWP = 21.2 mm, FW = 44.5 mm, PFCD = 5.9 mm, CH = 25.4 mm, MCPL = 48.5 mm, MCPH = 22.0 mm, P2ML = 26.2 mm, P2MW = 10.4 mm, pereopod 5, P5ML = 21.4 mm, and P5MW = 8.9 mm.

Additional material examined: Material is from 70 additional localities (n = 129 ♂, 86 ♀): Gamtoos River (Gamtoos drainage system), SAM A41019 (2 ♂), 33°44'50"S; 24°38'09"E, collected on 07.11.1984 by D. Coetzee; Groot River (Gourits drainage system), SAM A41060 (1 ♂, 1 ♀), 33°43'36"S; 24°37'14"E, collected on 09.11.1984 by D. Coetzee; Groot River (Gourits drainage system), SAM A41062 (1 ♀, 2 ♂), 33°41'29"S; 24°35'28"E, collected on 09.11.1984 by D. Coetzee; Groot River tributary near Ladismith (Gourits drainage system), SAM A41160 (2 ♀, 3 ♂), no GPS coordinate, collected on 31.10.1992 by Stewart, Hoenson, and Cook; and Gourits River (Gourits drainage system), SAM A41047 (1 ♂), SAM A41048 (1 ♀), SAM A41049 (1 ♀), SAM A41050 (1 ♀), SAM A41051 (1 ♀), SAM A41052 (2 ♂), SAM A41053 (2 ♂, 1 ♀), SAM A41054 (1 ♂, 2 ♀), 34°14'16"S; 21°45'45"E; 34°10'18"S; 21°44'44"E; 34°04'15"S; 21°43'09"E; 34°04'15"S; 21°43'09"E; 34°04'15"S; 21°43'09"E; 34°04'15"S; 21 43'09"E; 34°00'00"S; 21°38'23"E; and 33°54'28"S; 21°39'11"E (respectively), collected on 18.04.1985 and 18.11.1985 by D. Coetzee. I also examined specimens from tributaries of southern flowing drainages for which no genetic material was available for analysis. These additional specimens include another large drainage system, the Sundays River. Based on the biogeographical and drainage history of the region (Swartz *et al*., 2007), I postulate that *P. barbarai* sp. nov. occurs exclusively in southern flowing drainages, including the Sundays River. However, this assumption should be taken with caution, at least until resampling can be conducted. Though shown to be uninformative in this study, I also base this assumption on the morphological examination of the additional material that was collected from all southern flowing rivers (including the Sundays River drainage) that morphologically resembled *P. perlatus* s.s. However, because specimens of the Sundays River were not available for molecular studies, its taxonomic designation as *P. barbarai* sp. nov. remains provisional. Additional material: Baakensrivier,

SAM A41027 (1 ♂, 1 ♀), SAM A41037 (1 ♂, 1 ♀), SAM A41038 (3 ♂), 33°57'31"S; 25°33'37"E, 33°58'07"S; 25°35'50"E, and 33°57'31"S; 25°33'37"E (respectively), collected on 06.11.1984 by D. Coetzee; Baviaanskloof (Gamtoos drainage system), SAM A41018 (1 ♀), SAM A41035 (1 ♂, 1 ♀), 33°39'51"S; 24°23'23"E, collected on 10.11.1984 by D. Coetzee; Baviaanskloof (Geelhoutbos Reserve, Gamtoos drainage system), SAM A41134 (6 ♂, 3 ♀), no GPS coordinate, collected on 23.09.1992 by B. Stewart and L. Hoenson; Bietou River (Gamtoos drainage system), SAM A41014 (4 ♂, 1 ♀), SAM A41036 (1 ♀), 3°59'39"S; 23°17'46"E, collected on 22.02.1986 by D. Coetzee; Bot River (all collected by D. Coetzee), SAM A41015 (3 males) and SAM A41031 (2 ♀), 34°11'03"S; 19°13'24"E, collected on 27.09.1984; SAM A41030 (1 ♀), collected on 21.09.1984; SAM A41028 (1 ♂), SAM A41029 (1 ♂, 1 ♀), 34°09'24"S; 19°13'47"E, collected on 27.09.1984; SAM A41032 (2 ♂, 1 ♀), SAM A41033 (2 ♂), 34°12'02"S; 19°13'18"E, collected on 27.09.1984; SAM A41034 (1 ♂), 34°14'10"S; 19°12'59"E, collected on 27.09.1984; Duiwenhoeks River (all collected by D. Coetzee), SAM A41017 (4 ♂, 1 ♀), 34°06'49"S; 20°58'10"E, collected on 12.09.1985; SAM A41039 (1 ♀), SAM A41040 (3 ♂, 8 ♀), SAM A41041 (4 ♂, 5 ♀), 33°58'36"S; 21°01'54"E, 33°58'39"S; 20°59'41"E, 33°58'39"S; 20°59'41"E, collected on 11.09.1985; SAM A41042 (4 ♂, 1 ♀), SAM A41043 (2 ♂), 34°03'19"S; 20°57'53"E, collected on 11.04.1985; Goukamma, SAM A41022 (1 ♂), 34°01'30"S; 22°56'37"E, collected on 21.02.1986 by D. Coetzee; Grahamstown (Grey's reservoir), SAM A13387 (1 ♀), SAM A13387 (2 ♂, 1 ♀), 33°19'27"S; 26°31'42"E, collected on 16.10.1972 by R.E. Strobes; Groenland Nature Reserve (Eastern Cape), SAM A41135 (4 ♂), no GPS coordinate, collected on 24.11.1992 by B. Stewart and L. Hoenson; Hartenbos River (all collected by D. Coetzee), SAM A41023 (1 ♀), SAM A41076 (3 ♂, 1 ♀), SAM A41077 (2 ♂), SAM A41078 (1 ♂, 1 ♀), 34°05'58"S; 22°00'00"E, collected on 17.02.1986; SAM A41073 (3 ♂, 3 ♀), SAM A41074 (3 ♂), SAM A41075 (1 ♂, 1 ♀), 34°05'49"S; 21°59'45"E, collected on 17.02.1986; Highnoon (near Villiersdorp), SAM A41214 (1 ♀), no GPS coordinate, collected on 14.11.1994 by J. Hulley and L. Hoenson; Kafferkuilsriver (all collected on 20.11.1985 by D. Coetzee), SAM A41024 (1 ♂), 34°14'00"S; 21°17'29"E; SAM A41069 (2 ♂), SAM A41070 (2 ♂ 1 ♀), SAM A41071 (1 ♀), 34°08'00"S; 21°16'32"E; SAM A410722 (1 ♂), 34°05'30"S; 21°17'39"E; Kammanassi River (at Uniondale under bridge), SAM A41158 (3 ♂, 4 ♀), SAM A41170 (7 ♂, 4 ♀), no GPS coordinate, collected on 30.11.1992 by B. Stewart, L. Hoenson and P. Cook; Kleinbrak River, SAM A41080 (1 ♂), SAM A41081 (1 ♂), 34°03'24"S; 22°03'28"E, collected on 18.02.1986 and 18.11.1986 by D. Coetzee; Klipplaats (in river), SAM A13404 (1 ♀), 32°29'08"S; 26°56'56"E, no collection date,

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collected by J.C. Greig and C.T. Stuart; Kouga (Gamtoos drainage system), SAM A41020 (6 ♂, 5 ♀); SAM A41021 (1 ♂, 1 ♀), 33°40'17"S; 24°24'00"E, collected on 10.11.1984 by D. Coetzee; Leeukraal, SAM A41988 (2 ♂, 1 ♀), 34°07.439'S; 19°44.571'E, collected on 23.09.1995 by G. Ratcliffe; Stormsvlei/Avontuur (Riviersonderend), SAM A41987 (3 ♂, 1 ♀), 34°04.876'S; 20°05.161'E, collected on 23.09.1995 by G. Ratcliffe; Sundays River (all collected on 08.04.1986 by D. Coetzee), SAM A41056 (1 ♀), SAM A41057 (1 ♀), SAM A41058 (1 ♂, 1 ♀), 33°34'55"S; 25°40'31"E; SAM A41059 (1 ♂, 1 ♀), 33°25'41"S; 25°26'27"E; Swartkops, SAM A41065 (2 ♀), 33°48'37"S; 25°31'56"E, collected on 09.04.1986 by D. Coetzee; Tarkastad, SAM A13398 (1 ♂), 32° 13'57"S; 26°16'42"E, collected on 07.12.1972 by R.T. Philips; and Uilkraals River, SAM A41016 (3 ♂, 1 ♀), SAM A41046 (1 ♀), 34°31'38"S; 19° 32'22"E, no collection date, collected by D. Coetzee.

Description and diagnosis: Potamonautes barbarai sp. nov. was diagnosed on the basis of molecular findings rather than morphology. The species has a carapace depth (CH/CL) of 0.59 mm and a posterior margin width (CWP/CL) of 0.49 mm. There are no visibly discrete morphological characters to separate this species from *P. perlatus* s.s. and the gonopods are also similar (Fig. 7D-F) to those of *P. perlatus* s.s. (Fig. 7A-C).

Distribution: *Potamonautes barbarai* sp. nov. occurs in large rivers and is rarely found in first order streams. This species is endemic to southern flowing drainages (Gamtoos River and Gourits River) and their tributaries. The Cape Fold Mountains have been extensively sampled for freshwater crabs, and the species has not been recorded elsewhere. However, based on the biogeographical history of the region, I presume that the specimens collected from other southern flowing drainages, up to Sundays River, are also *Potamonautes barbarai* sp. nov. Accordingly, this species is endemic to the south flowing drainages of the Cape Fold Mountains and parts of the Eastern Cape, including the Sundays River drainage system (provisional designation).

Remarks: The species bears a striking morphological resemblance to *P. perlatus sensu stricto* (clade 2, Fig. 2), however the molecular systematics results revealed otherwise. Moreover, when considering diagnostic character states between the three lineages, particularly the gonopods (Fig. 7 (A- I), no morphological differences were found.

Etymology: *Potamonautes barbarai* sp. nov. is named after Prof. Barbara A. Stewart in recognition of her considerable contributions to the taxonomy of freshwater crabs and other freshwater crustaceans (amphipoda, decapoda, and isopoda) in South Africa.

Conservation: Up until the present study, *Potamonautes barbarai* sp. nov. was taxonomically designated as *P. perlatus* s.l., which is "widespread", occurring from Namibia

to the Eastern Cape. As a widespread species, *P. perlatus* s.l. is listed as Least Concern (Cumberlidge, 2013). The species is likely to face similar threats as *P. perlatus* s.s., i.e. habitat loss and degradation (Cumberlidge, 2013). In the 71 localities that *P. barbarai* sp. nov. was recorded, only one was in a conservation area (Groenland Nature Reserve, Eastern Cape). Pending the demarcation of this species' distribution range and abundance studies, the conservation status of the species is currently Data Deficient.

POTAMONAUTES BARNARDI SP. NOV. (CLADE 1B, FIG. 2)

MATERIAL EXAMINED

Holotype: Klein River (Breede River drainage system), SAM A41013 (1 ♂), 34°21'54"S; 19°38'19"E, collected on 18.04.1985 by D. Coetzee. This haplotype is endemic to the Breede River drainage system. Morphological measurements of the holotype are as follows: CL = 36.9, CWW = 54.6, CWP = 18.2, FW = 37.5, PFCD = 5.2, CH = 22.8, MCPL = 43.0 MCPH = 17.2, P2ML = 23.2, P2MW = 8.10, P5ML = 20.0 and P5MW = 7.7.

Additional material examined: Material is from nine localities (n = 20 ♂, 11 ♀). With the exception of Tunnel Terminals, all additional material examined is from the Breede River drainage system: Bainskloof (Wellington side), SAM A11075 (1 ♀), no GPS coordinate, collected on 29.09.1962 by Grundley; Bonnievale, SAM A41165 (1 ♂, 1 ♀), no GPS coordinate, collected on 04.04.1994 by L. Hoenson and M. van der Merwe; Buffelsjag River (tributary, Swellendam), SAM A41144 (5 ♂, 4 ♀), no GPS coordinate, collected on 30.10.1992 by B. Stewart, L. Hoenson, and M. van der Merwe; De Hoop, SAM A41203 (1 ♂), no GPS coordinate, collected on 05.05.1994 by B. Cook, L. Hoenson; Klein River, SAM A41012 (1 ♂), 34°21'54"S; 19°38'19"E, collected on 18.04.1985 by D. Coetzee; Klein River, SAM A41083 (1 ♂), 34°24'55"S; 19°32'30"E, collected on 18.04.1985 by D. Coetzee; and Robertson (river near Montagu), SAM A19367 (1 ♂), no GPS coordinate, collected in March 1985 by B. Badenhorst; Tradouw Pass (between Barrydale and Swellendam), SAM A41161 (10 ♂, 4 ♀), no GPS coordinate, collected on 01.09.1992 by B. Stewart, L. Hoenson, M. van der Merwe, and Anderson; Tunnel Terminals (LaMotte Forest, Berg River drainage system), SAM A43912 (1 ♀) and SAM A44979 (4 ♂), 34°57'00"S; 19°04'00"E and 33°54'00"S; 19°03'00"E, collected on 04.12.1995 and 05.12.1995 (respectively) by B. Stewart, L. Hoenson, M. van der Merwe, and Cochrane.

Description and diagnosis: *Potamonautes barnardi* sp. nov. was diagnosed based on molecular results. The carapace depth of *P. barnardi* sp. nov. (CH/CL) = 0.61 mm, while the

posterior margin width (CWP/CL) = 0.49 mm. There are no distinct morphological characters to separate this species from *P.barbarai* sp. nov. and *P. perlatus* s.s. and, perhaps significantly, the adult gonopods of all three taxa are identical, as also illustrated in the photographs of the gonopods (Fig. 7G-I).

Distribution: This species occurs in tributaries of the Breede River and is also found in the Berg River at Tunnel Terminals (LaMotte Forest). A fine scale sampling survey, particularly of the area where the Breede River and Berg River are thought to have historically been connected (du Toitskloof), is required to delineate the distribution range of the species and to ascertain its extent of occurrence in the Berg River drainage system.

Remarks: *Potamonautes barnardi* sp. nov. is morphologically identical to *P.barbarai* sp. nov. and *P. perlatus* s.s. Diagnostic morphological character states, specifically the gonopods (Fig. 7 (A- I), are morphologically indistinguishable.

Etymology: *Potamonautes barnardi* sp. nov. is named after Dr. Keppel H. Barnard whose studies (Barnard, 1935, 1950) established the presently recognized distributional range of *P. perlatus* s.l., which is refined here in the present study.

Conservation: The conservation status of *P. barnardi* sp. nov. is Data Deficient. Identical to *P. barbarai* sp. nov., *P. barnardi* sp. nov. was taxonomically designated as the "widespread" *P. perlatus* s.l., which according to Cumberlidge (2013) is a Least Concern conservation priority. However, the Breede River Valley has been earmarked as a priority conservation area, particularly the freshwater (dams and rivers) systems (Cumberlidge, 2011). For example, *Pseudobarbus burchelli* (the only indigenous fish species in the Tradouw catchment) is restricted to a single tributary of the Breede River drainage system and is listed as Critically Endangered as a result of excessive water abstraction from farming, habitat destruction and pollution (Snoeks *et al*., 2011), which are among the biggest threats faced by freshwater crabs (Cumberlidge, 2011). The Breede River Valley has limited winter and spring rainfall compared to the rest of the Western Cape, which is a winter rainfall region (Schulze, 1997). Therefore, the possible drying of some streams may lead to a loss of habitat for *P. barnardi* sp. nov., which is why a fine scale and complete survey of the populations of this species is necessary.

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Table 1 Sampling localities (including geographical position) for *Potamonautes perlatus* separated by drainage systems in the Eastern and Western Cape provinces of South Africa. *N* represents the number of specimens per locality.

Drainage system	Collection locality (position on map, Fig.1)	*N*	Longitude	Latitude
Olifants River	1 Olifants	1	-31.901264	18.604317
	2 Clanwilliam	2	-32.208492	18.969386
	3 Kriedouwkrans	3	-32.364736	18.954033
	4 Boontjieskloof	2	-32.558064	19.113869
	5 Citrusdal	4	-32.562367	19.318369
	6 Boesman	3	-32.515286	19.376333
Cape Peninsula	7 Liesbeeck	4	-34.190519	18.416639
	8 Tokai	4	-34.000817	18.387022
Eerste River	9 Stellenbosch	5	-33.95183	18.90585
Berg River	10 Paarl	3	-33.829569	19.054844
	11 Tunnel Terminals	4	-33.730908	19.118247
Breede River	12 Bainskloof	3	-33.589581	19.129067
	13 Robertson	4	-33.832397	19.869556
	14 Klein River	1	-34.426492	19.511075
	15 Bonnievale	1	-33.945622	20.102786
	16 De Hoop	4	-34.350272	20.596106
Gourits River	17 Touws River	1	-33.339392	20.014000
	18 Nels River	5	-33.487644	21.433878
	19 Vette River	5	-34.019822	21.216314
	20 Groot River	5	-33.670611	21.165992
	21 Huis River	5	-33.500542	21.600689
	22 Dwyka River	5	-33.087350	21.570931
	23 Vlei River	4	-33.551833	21.884861
	24 Prince Albert	5	-33.174269	22.027631
	25 Swartberg Pass	4	-33.324906	22.047717
Gamtoos River	26 Smithskraal	5	-33.653444	24.354361
	27 Kleinplaats	5	-33.636917	24.453944
	28 Poortjies	3	-33.658481	24.531992
	29 Sand River	4	-33.689811	24.591153
	30 Bosdorp	5	-33.669383	24.574017
	31 Andrieskraal	5	-33.742831	24.629981
	32 Patensie	4	-33.776225	24.809872
	33 Hankey	4	-33.841831	24.861986
Total		122		

Table 2 Model parameters, as obtained in jModelTest v. 2.1.3, for each locus used in this study.

Gene fragment	Base pairs	Haplotypes	Haplotype diversity (*hd*)	Parsimony informative sites	Polymorphic variable sites	Model	Base pair frequencies (%)	Gamma (G) distribution shape	Proportion invariable sites (I)
16S rRNA	370	24	0.8970	23	33	TPM3uf + I + G (- ln L = 841.32; AIC = 2188.64)	A = 38.05	0.29	0.119
							C = 8.11		
							G = 18.24		
							T = 35.61		
28S rRNA	577	10	0.8185	7	58	TVM + G (-ln L = 1606.60; AIC = 3365.21)	A = 13.67	0.46	N/A
							C = 32.88		
							G = 33.90		
							T = 19.55		
COI	628	34	0.8987	141	202	TPM1uf + G (- ln L = 2933.57; AIC = 6371.14)	A = 29.74	0.36	N/A
							C = 20.43		
							G = 13.91		
							T = 35.93		

Table 3 AMOVA results for the population delineations within *Potamonautes perlatus* for the COI haplotypes.

Clades	Variation among localities (%)	Variation within localities (%)	Φ_{ST} ($p < 0.0001$)	Fu's F_S ($p > 0.05$)	Tajima's D ($p > 0.05$)
Overall localities	84.78	15.22	0.85	0.43	9.08
Clade 1	63.59	36.41	0.64	0.37	- 0.30
Clade 2	95.07	4.93	0.95	0.05	- 0.15

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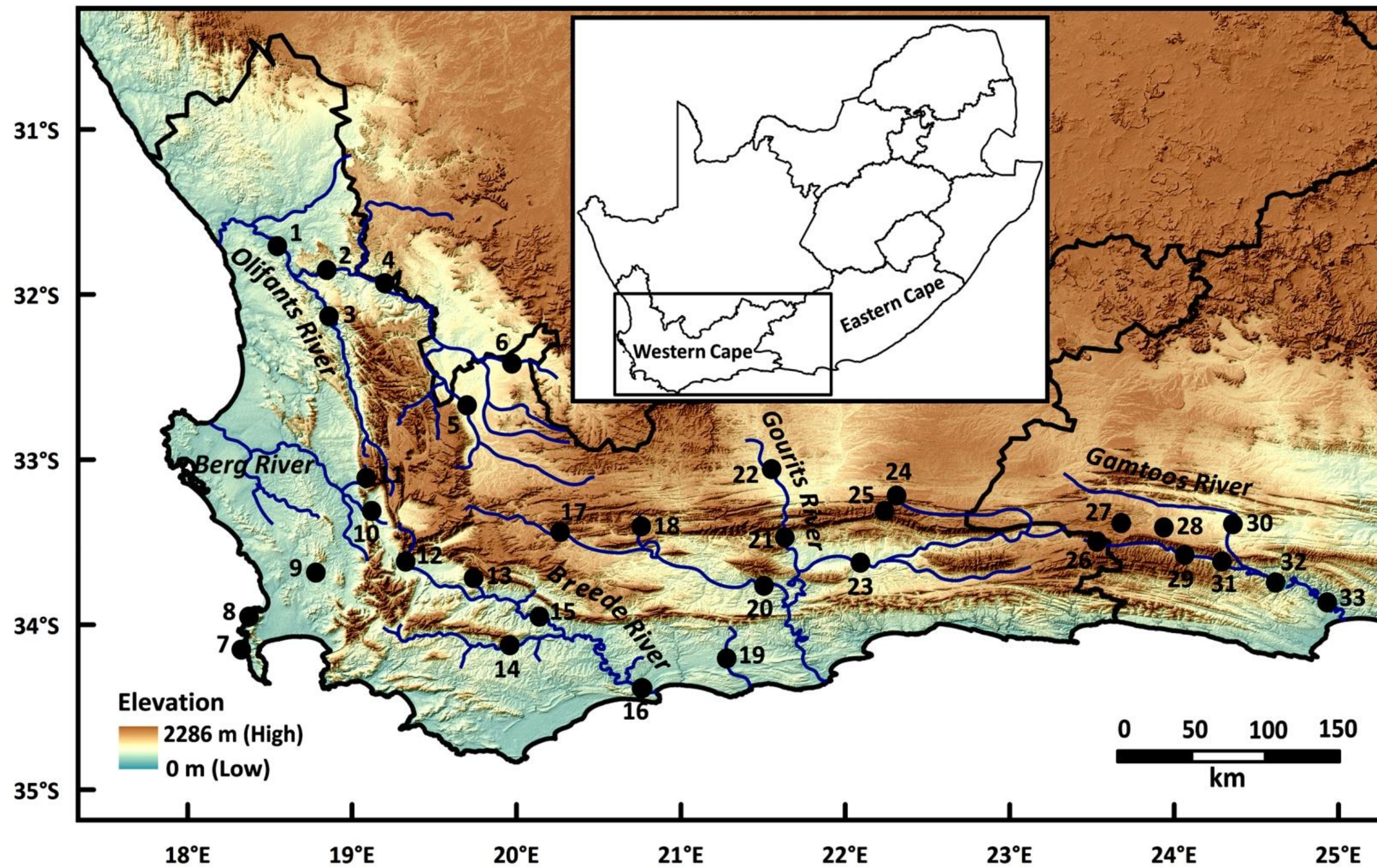

Figure 1 Sampling localities for *Potamonautes perlatus* s.l. along rivers occurring on the Cape Fold Mountains. The black circles with adjacent numbers represent localities (Table 1).

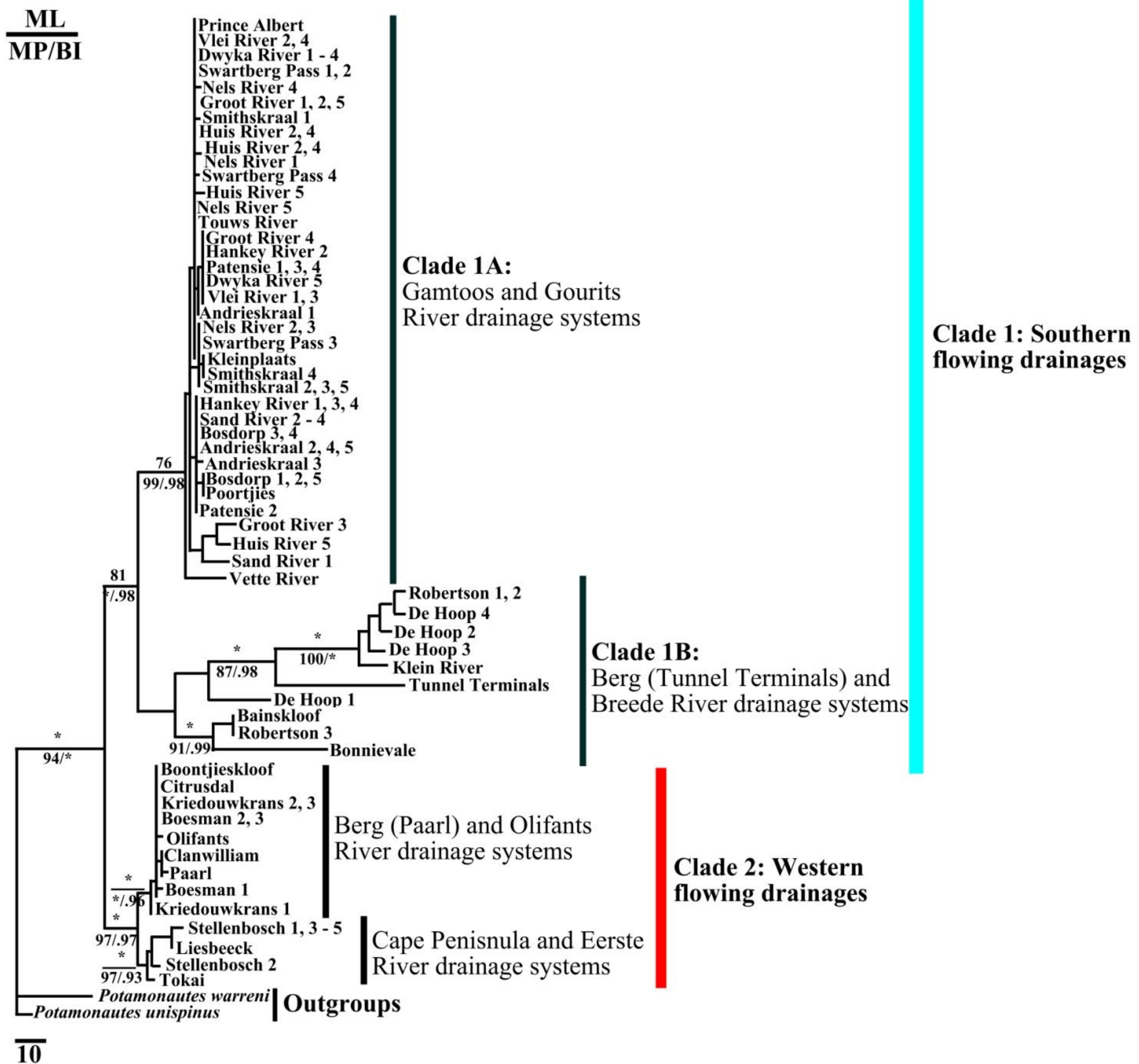

Figure 2 A maximum likelihood phylogram of the combined mtDNA sequences for the phylogenetic reconstruction of *Potamonautes perlatus* s.l. The ML node support is shown by bootstrap values at the top of each branch (only values above 75% are shown). The values at the bottom of the branches are bootstrap values for MP/posterior probabilities for BI (the * indicates no support). The numbers next to each taxon name represents the individual specimens, no numbering is given to taxa where the entire population occurs on one branch.

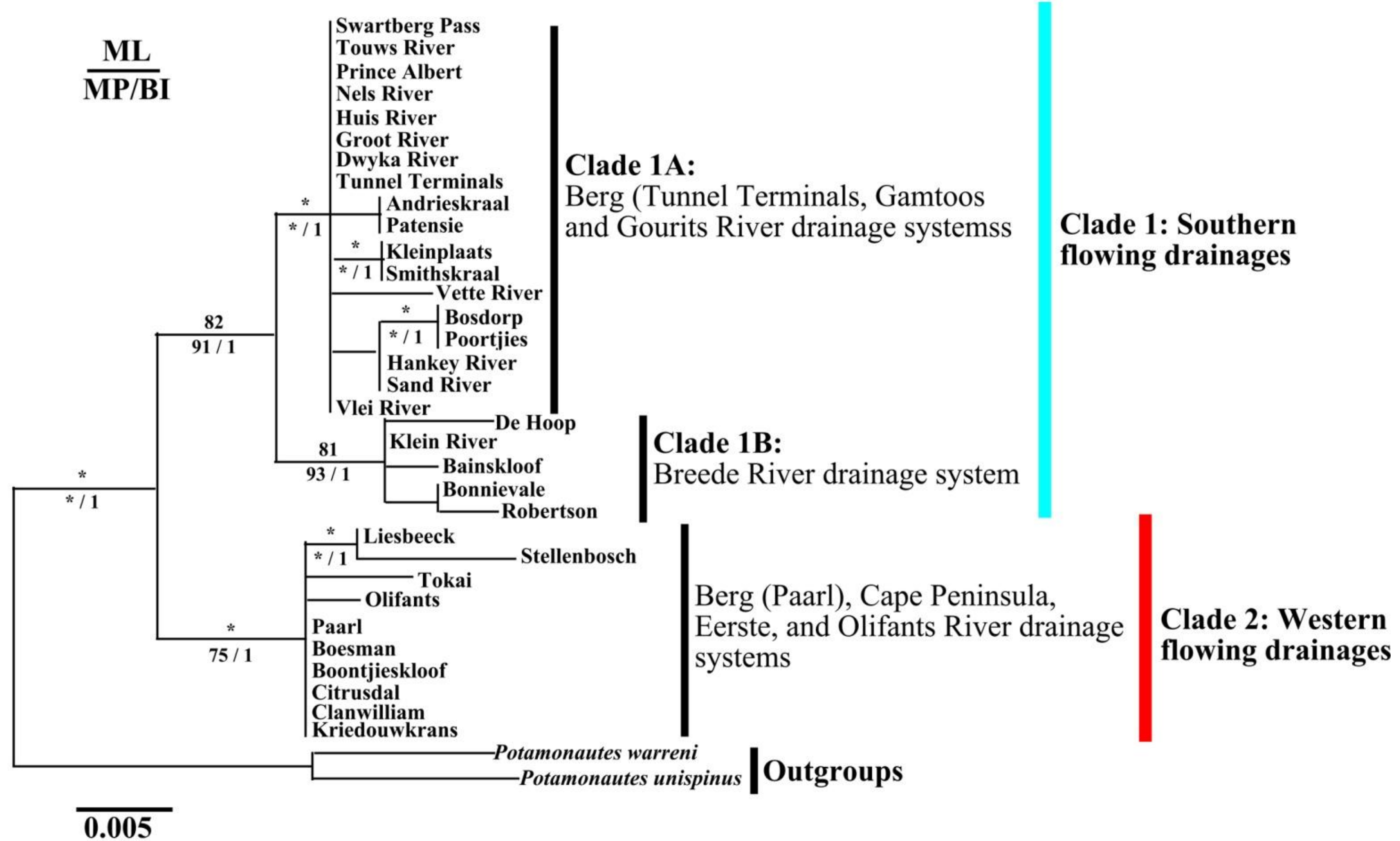

Figure 3 A consensus Bayesian inference phylogram of *Potamonautes perlatus* s.l. from the combined nuDNA (28S rRNA) and mtDNA (COI and 16S rRNA) datasets as well as outgroups. Node support values are shown by ML bootstrap values above and maximum parsimony (MP) / posterior probabilities of Bayesian inference (BI) below. Low bootstrap support (< 75% for MP and ML) and posterior probability (< 0.95 for BI) values are not shown. The * is indicative of nodes that are not statistically supported.

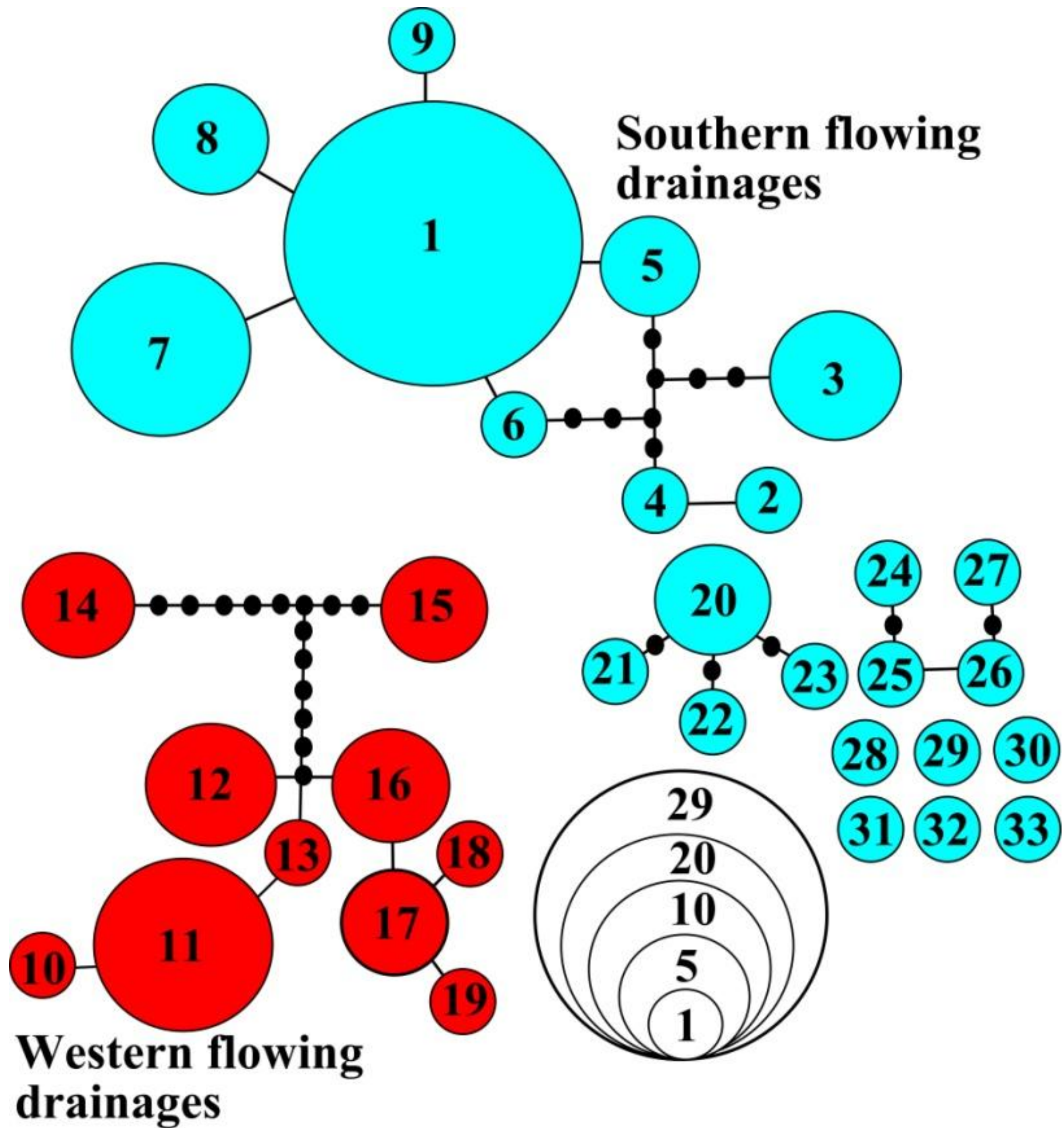

Figure 4 A 95% parsimony haplotype network of *Potamonautes perlatus* s.l. populations for the COI gene. The numbers inside the circles correspond to the haplotype number and the size of the circles represents the number of individuals in a haplotype. The black solid circles are indicative of extinct or unsampled haplotypes.

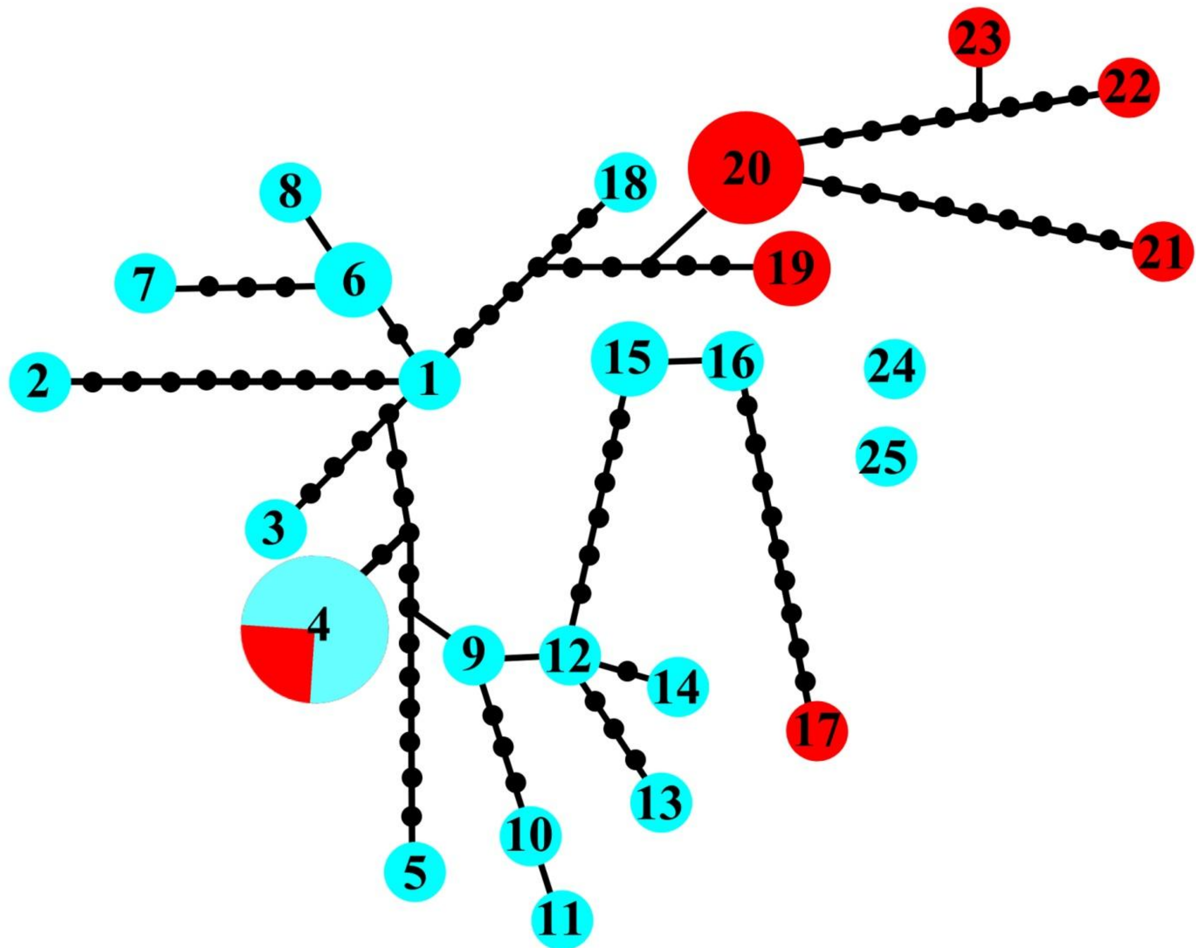

Figure 5 A 95% parsimony haplotype network of 28S rRNA for *Potamonautes perlatus* s.l. The numbers in the circles represent the haplotype number, while the colour of the circles represent the different clades (see Figs. 2 and 3). The size of circles represents the frequency of the haplotype. 1 = Groot River, 2 = Smithskraal, 3 = Bainskloof, 4 = Olifants River, Sand River, Andrieskraal, and Touws River, 5 = De Hoop, 6 = Vette River and Robertson, 7 = Kleinplaats, 8 = Bonnievale, 9 = Bosdorp, 10 = Poortjies, 11 = Klein River, 12 = Nels River, 13 Swartberg Pass, 14 = Dwyka River, 15 = Prince Albert and Vlei River, 16 = Huis River, 17 = Tokai, 18 = Hankey River, 19 = Liesbeeck and Stellenbosch, 20 = Paarl, Kriedouwkrans, and Clanwilliam, 21 = Boesman, 22 = Citrusdal, 23 = Boontjieskloof, 24 = Patensie, 25 = Tunnel Terminals.

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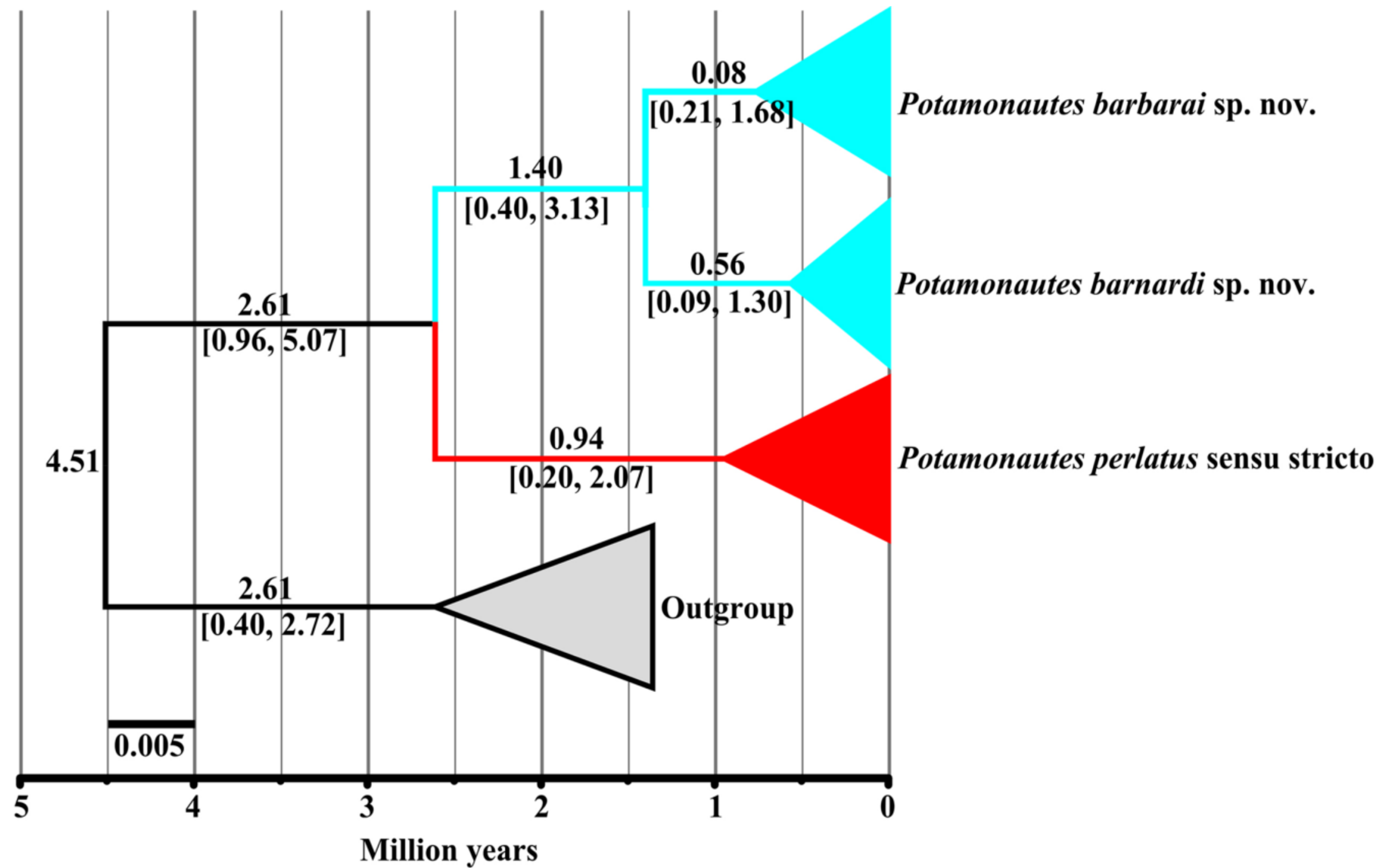

Figure 6 Divergence time estimations of *Potamonautes perlatus* s.l. Values above branches are the divergence dates in million years and the values in square brackets are the 95% highest posterior density (HPD) values.

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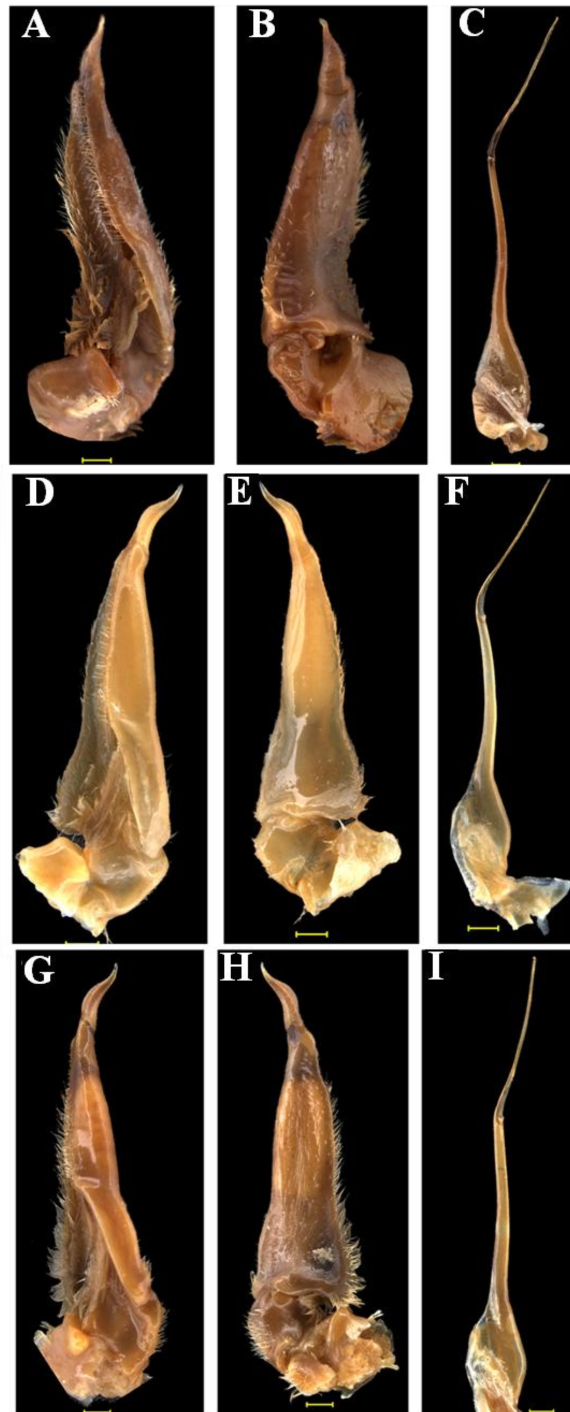

Figure 7 A – C *Potamonautes perlatus* s.s male neotype (SAM A45755): A = left gonopod 1, anterior view: B = left gonopod 1 posterior view; C = left gonopod 2 posterior view; **D – F** *Potamonautes barbarai* sp. nov. male holotype (SAM A41061): D = left gonopod 1, anterior view; E = left gonopod 1 posterior view; F = left gonopod 2 posterior view; and **G – I** *Potamonautes barnardi* sp. nov. male holotype (SAM A41013): G = left gonopod 1, anterior view; H = left gonopod 1 posterior view; I = left gonopod 2 posterior view; scale bars = 1.0 mm.

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CHAPTER 3

A COALESCENT MULTILOCUS SPECIES DELIMITATION APPROACH DEMONSTRATES WIDESPREAD CRYPTIC DIFFERENTIATION WITHIN TWO MOUNTAIN-LIVING FRESHWATER CRAB LINEAGES *

ABSTRACT

The presence of cryptic lineages presents major challenges for evolutionary, ecological, and conservation studies, particularly where cryptic lineages remain undiscovered. Freshwater crabs are known to harbour cryptic diversity, in most cases with no morphological differences, which may hamper the progression of studies on their evolutionary histories and biogeography. In this study, I used a multilocus (mt- (n = 3) and nuDNA (n = 3)) Bayesian species delimitation method, as implemented in the program Bayesian phylogenetics and phylogeography (BP&P), to examine cryptic species diversity within a freshwater crab species complex (*Potamonautes clarus* / *P. depressus*). I sampled 25 high-lying rivers occurring in the Tugela and uMkomazi River drainage systems (Drakensberg Mountain range, KwaZulu-Natal Province, South Africa). While previous research delimited between two and five lineages within this species complex, the results showed that there were at least eight lineages (six novel, and two described as *P. clarus* / *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 approximately 10.3 Mya up until the Holocene (0.11 Mya), with each delimited lineage strongly supported by divergence time estimations. The discovery of six novel lineages from a seemingly restricted distribution ranges has conservation implications. However, many conservation planning strategies are focussed on freshwater vertebrates (fish and amphibians). By conducting a fine-scale phylogenetic survey using invertebrates, this study provides a platform for the inclusion of freshwater invertebrates for future conservation assessments.

INTRODUCTION

Accurately delineating species boundaries is central in evolutionary and ecological studies, specifically in studies involving the assessment of biodiversity and recognizing areas

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of conservation priority (Sites & Marshall, 2003; 2004; Agapow *et al*., 2004; Witt *et al*., 2006; Bickford *et al*., 2007; Carstens & Dewey, 2010; Camargo *et al*., 2012; Camargo & 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 & Leaché, 2011; Carmago & Sites, 2013). 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 display the inability to morphologically accommodate the evolutionary processes that have occurred during the speciation process within a 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 *et al*., 2009).

While molecular data are informative with regards to the hierarchical relatedness and the relative rates of evolution between species, they can still provide poorly resolved phylogenetic trees because of the presence recently diverged species (Brito & Edwards, 2009; Yang & 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 & Marshall, 2003, 2004; Carmago & Sites, 2013). Moreover, traditional methods of species delimitation consist of analyzing multiple loci in concatenated datasets, forming a supermatrix, under the assumption that the different loci share the same species tree topology (Kubatko & Degnan, 2007; Degnan & Rosenberg, 2009; Heled & Drummond, 2010). However, it was demonstrated that species tree inference using concatenated datasets had some limitations (Kubatko & Degnan, 2007; Degnan & Rosenberg, 2009) and that it involved the subjective separation of species boundaries (Hey, 2009) without statistical exploration (Knowles, 2009; Leaché & Fujita, 2010; Carmago & Sites, 2013; Rannala & Yang, 2013). Hence, coalescence-based species tree inference using independent multi-loci and Bayesian analysis have become widely accepted as robust methods for delineating species (Rannala & Yang, 2003; Liu & Pearl, 2007; Liu, 2008; Degnan & Rosenberg, 2009; Liu *et al*., 2009; Edwards, 2009; Kubatko *et al*., 2009; Heled & Drummond, 2010; Knowles & Kubatko, 2010; O'Meara, 2010; Yang & Rannala, 2010; Ence

& Carstens, 2011; Carmago & Sites, 2013; Rannala & Yang, 2013). The coalescent in the Bayesian approach acts as a prior distribution for the gene tree (Heled & Drummond, 2010). Estimating species trees (barriers for gene flow) with this approach has been reported to outperform data concatenation-based species tree inferences (Kubatko & Degnan, 2007; Liu *et al*., 2008; Kubatko *et al*., 2009; Heled & Drummond, 2010). One of the reasons for this is that the coalescent species tree model autonomously infers each locus genealogy and assumes that any incongruencies between gene trees results from incomplete lineage sorting and that each delimited cryptic species is panmictic (Carstens & Dewey, 2010; Heled & Drummond, 2010; Carmago & Sites, 2013; Rannala & Yang, 2013). Therefore, in conjunction with morphology, it is also essential to evaluate multiple characteristics, including multiple loci and / or genomes, to increase the likelihood of detecting recent lineage separation and to obtain robust evidence of lineage sorting among cryptic species (Heled & Drummond, 2010). Accurately delimiting cryptic species will have 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 occurring in various types of habitats (e.g. Pfenninger *et al*., 2003; Hebert *et al*., 2004; Fouqueta *et al*., 2007; Murray *et al*., 2008; Lohman *et al*., 2010; Funk *et al*., 2012; Knee *et al*., 2012; Paupério, 2012; Pedraza-Lara *et al*., 2012; Cavers *et al*., 2013; Lemme *et al*., 2013, McFadden & van Ofwegen, 2013; Smith *et al*., 2012; 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, they 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*., 2007; Jesse *et al*., 2010; Padraza-Lara *et al*., 2012, Phiri & Daniels, 2014).

Freshwater crabs (Decapoda: Brachyura) are morphologically conserved in key taxonomic factors, which often hampers cryptic species delimitation (Daniels *et al*., 2006a). 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; Daniels *et al*., 1999a, 2001, 2003, 2006b). As such, the systematics of the genus is obscured (Daniels *et al*., 2003, 2006), 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). Gouws *et al*. (2000) previously differentiated between the two species. However, these conclusions (Gouws *et al*., 2000; Daniels *et al*., 2003) were based on allozyme data and a single mitochondrial DNA (16S rRNA) marker. The use of allozyme data in phylogenetic studies is outdated and cannot be used for phylogeography studies because similarities between alleles in allozyme data are indeterminate, and those similarities or differences are often influenced by environmental conditions (Hedgecock, 1986). 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, and specifically 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 & Barrowclough, 2008; Edwards & Bensch, 2009) and that it does not take random lineage sorting into account (Heled & Drummond, 2010). Moreover, it has also been demonstrated that mtDNA on its own (without the addition of nuDNA loci) can be misleading in freshwater crab phylogenetic studies (Barber *et al*., 2012). This leaves the question as to whether the five lineages that were recognized by 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 I 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 distribution range (Drakensberg Mountains, KwaZulu-Natal, South Africa). Moreover, I 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 (n = 117) were collected from 25 localities (Table 1, Fig. 1) in the Drakensberg Mountain range (KwaZulu-Natal Province, South Africa) in August and September 2011. I sampled the Tugela (n = 10) and uMkomazi River (n = 15) drainage systems. According to Daniels *et al*. (2003), out of the nine sampled localities, *P. clarus* primarily occurs in the northern parts of the Tugela River drainage system (three localities), while the intermediate / cryptic lineages are found in central parts of the same drainage system (four localities). The remaining two localities were found to comprise *P. depressus* populations. However, it is important to note that Daniels *et al*. (2003) only sampled one locality in the southern-most bounds of the Tugela drainage system for *P. depressus*, leaving a big sampling gap between the intermediates and the "true" *P. depressus*. Moreover, the second locality comprising *P. depressus* was sampled in the uMkomazi River drainage system, with no other localities / rivers 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 and one in the uMkomazi drainage), with a wide sampling gap between the sampling localities within the uMkomazi drainage system.

Ox-heart baited lines were used to attract the crabs, after which they were killed by freezing for 24 hrs prior to tissue extraction. One pereopod (walking leg) was broken off from each specimen and preserved in 95% ethanol for DNA analysis.

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. I amplified three mitochondrial partial gene fragments (12S rRNA, 16S rRNA, and COI) and three nuclear DNA markers (28S rRNA, DecapANT, and PEPCK) – see Table 2 for the list of primer pairs and the PCR profiles for each of the markers. All of these markers have been widely applied in phylogenetic studies at various crustacean (including decapod) taxonomic levels (Daniels *et al*., 2002, 2006a, b; Klaus *et al*., 2006; Shih *et al*., 2006; Tsang *et al*., 2008; Teske *et al*., 2009; Jesse *et al*., 2010; Daniels, 2011; Barber *et al*., 2012). For COI and 16S rRNA I amplified 5 specimens per locality, while only two specimens per locality were amplified for

the other four loci. This was done to accommodate the various analytical methods, specifically for obtaining the species tree using the COI and 16S rRNA sequence data. For the nuDNA markers, I amplified one or two specimens per locality based on the COI / 16S tree topology.

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 to Macrogen Europe for sequencing (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). Coding gene fragments (COI and PEPCK) were translated to amino acids (EMBOSS-Transeq, http://www.ebi.ac.uk/emboss/transeq/) and no stop codons were detected, indicating that all sequences were valid.

Sequence diversity and population relationships

The basic sequence statistics (i.e. number of haplotypes (h), haplotype diversity (*Hd*), number of variable (polymorphic) and parsimony informative sites, and neutrality tests – Tajima's *D* and Fu's F_s) were calculated in DnaSP v. 5.10 (Librado & Rozas, 2009). Relationships between populations or haplotypes were examined for the COI genetic marker. In this study, COI is the most variable mitochondrial gene, and is therefore suitable for investigating population structure. TCS v. 1.21 (Templeton & Sing, 1992; Crandall & Templeton, 1996) was used to construct a 95% parsimony haplotype network of the dataset, where gaps were treated as missing data. In addition to the parsimony haplotype network, I constructed a minimum spanning network (MSN) with distances between haplotypes obtained from Arlequin v. 3.5.1.2 and viewed in HapStar v. 0.7 (Teacher & Griffiths, 2011). Uncorrected between clade sequence "p" distances were calculated in MEGA5 v. 2.2 (Tamura *et al*., 2011)

Phylogenetic reconstructions

I first reconstructed the phylogeny (gene trees) of the *P. clarus* / *P. depressus* species complex using the concatenated mtDNA (cmtDNA: 12S rRNA, 16S rRNA, and COI), concatenated nuDNA (cnuDNA: 28S rRNA, DecapANT, and PEPCK), and the combined cmtDNA and cnuDNA 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*., 2003). I first conducted a BI analysis on the combined 16S rRNA and COI dataset (n = 117 sequences per locus, n = 2 to 5 specimens per locality), in BEAST v. 2.0.2 (required for the species delimitation analysis, explained below). The topology and posterior probabilities (nodal support) were corroborated with a BI analysis using MrBayes v. 3.2.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003; Ronquist *et al*., 2012). All other BI phylogenetic reconstructions were conducted in MrBayes only. Consensus BI trees were constructed for all datasets, i.e. cmtDNA, cnuDNA, and combined cmtDNA and cnuDNA, in a partitioned analysis using best-fit substitution models obtained for each gene separately from jModelTest v. 2.1.3 (Posada, 2008) (see Table 3 for model information). Analyses were conducted with four Markov Chain Monte Carlo (MCMC) simulations which were run for 5 x 10^6 generations, with each chain starting from a random tree and parameters sampled every 5 x 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 *et al*., 2009). Only branches with a posterior probability (p*P*) support ≥0.95 were considered statistically supported.

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

SPECIES TREE, SPECIES DELIMITATION, AND DIVERGENCE TIME ESTIMATION

I utilized the multispecies coalescent species tree reconstruction method in *BEAST (as implemented in BEAST v. 2.0.2) to deduce the species tree topology from the multiple gene trees of the multilocus dataset (Drummond *et al*., 2002, 2009, 2012a, b; Heled & Drummond, 2010). *BEAST 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 & Drummond, 2010; Drummond *et al*., 2012a, b). This method attributes incomplete lineage sorting, and not gene flow, as the main cause of incongruence among multiple gene trees (Heled & Drummond, 2010; Drummond *et al*., 2012a, b). Compared to other species delimitation methods, *BEAST has been demonstrated to be more accurate even when used on fast evolving lineages or shallow phylogenies (Drummond & Rambaut, 2007; Heled & Drummond, 2010; McCormack *et al*., 2011). However, to construct the topology of the species tree, *BEAST requires that species tree is given *a priori*. I therefore used the results from the phylogenetic reconstructions (BI) of the mtDNA (only 16S rRNA and COI) (Yang & Rannala, 2010) as well as the 95% parsimony haplotype network to define groups to be mapped as species. From the phylogenetic reconstructions, I identified eight well-supported clades, corroborated by the haplotype network. From these identified clades I 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 x 10^7 iterations, with chains and trees sampled every 5 x 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 & 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 visualized in FigTree v. 1.4 (Drummond *et al*., 2009). The obtained tree was then utilized 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), I conducted a multilocus coalescent species delimitation analysis implemented in the C-program, Bayesian phylogenetics and phylogeography (BP&P) v. 2.2 (Rannala & Yang, 2003; Yang & 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 & 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 ($\tau 0$) was assigned the gamma prior $G(2, 1000)$, while the other divergence time parameters were assigned the Dirichlet prior (Yang & Rannala, 2010: equation 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, 2009, 2012a, b; Heled & 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 in the mitochondria. While phylogenetic dating using shallow phylogenies and slow evolving or less variable genetic markers has minimal influence on the posterior (Brown & Yang, 2010), I 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*., 2006b; 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 (SD) 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}; SD = 1.94×10^{-9}) for 16S rRNA. The input (xml) file was created in BEAUti v.2.0.2 (Drummond & Rambaut, 2007; Drummond *et al*., 2012a, 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 & Drummond, 2012). Substitution models (obtained from jModelTest, Table 3) and clock rates were unlinked, whereas the nuDNA trees were unlinked

for analysis. Four MCMC chains were run four times for 200 x 10^6 iterations, with chains and trees sampled every 20 x 10^3 generations. An effective sample size (ESS) >200 for each parameter (visualized in Tracer v. 1.5) verified the convergence of the four combined chains (in LogCombiner v. 2.0) (Drummond & Rambaut, 2007; Drummond *et al.*, 2009). 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 visualized in FigTree v. 1.4 (Drummond *et al.*, 2009).

RESULTS

SEQUENCE DIVERSITY AND POPULATION RELATIONSHIPS

The basic statistics for all the gene fragments are shown in Table 4. The COI gene fragment was the most informative of the mtDNA markers, while 28S rRNA was the most informative of the nuDNA markers. PEPCK was the least informative genetic marker, with six variable sites and only one parsimony informative site. TCS collapsed the 117 COI sequences to 55 haplotypes, with nine distinct groupings and two singleton haplotypes – haplotypes 54 (Gudufalls) and 55 (Highmoore Nature Reserve) (Fig. 2), and haplotype frequencies ranging from 1 to 14 per haplotype (Table 3). Haplotype sharing between the two drainage systems only occurred between specimens from Kamberg (uMkomazi drainage) Highmoore Nature Reserve (Tugela drainage).

PHYLOGENETIC RECONSTRUCTIONS

Concatenated mtDNA (12S rRNA, 16S rRNA, and COI)

Phylogenetic reconstructions of the individual loci (not shown) from both the BI and ML analyses revealed 5 well-supported congruent major population groupings for both COI and 16S rRNA, with varying nodal support for individual sub-clades (nine for COI and eight for 16S rRNA). While also congruent with the COI and 16S rRNA topologies, 12S rRNA (the least variable mtDNA marker of the three) supported four major clades with six sub-clades (see Fig. 3 for the cmtDNA dataset topology).

The cmtDNA (12S rRNA, 16S rRNA, and COI) supported a monophyletic grouping of four clades (seven subclades: 1A, 1B, 1C, 2A, 2B, 3, and 4) (Fig. 3), with uncorrected between clade sequence “p” distances ranging from 4.1% to 15.2% (Table 5). Clade 1 comprised specimens collected from the uMkomazi drainage system (southern Drakensbeg)

(ML = 90%, p*P* = 1.00). However, this clade also included specimens from three localities occurring in the Tugela drainage system, i.e. Cathedral Peak (b) (clade 1A) and Highmoore Farm and Highmoore Nature Reserve (b) (ML = 90%, p*P* = 0.99; clade 1C, Fig. 3).

Clade 2 only comprised specimens from the Tugela drainage system as retrieved by Gouws *et al*. (2000) and Daniels *et al*. (2003) and was only supported by BI analysis (ML <75%, p*P* = 0.99). Clade 2A (ML = 100%, p*P* = 1.00) was made up of Monks Cowl (b and 2a) and Highmoore Nature Reserve (a). The third clade (clade 3) comprised Cathedral Peak and Injisuthi (a) (ML = 93%, p*P* = 1.00). Clade 4 only comprised specimens from Injisuthi (b) (ML = 100%, p*P* = 1.00).

Concatenated nuDNA (28S rRNA, DecapANT, and PEPCK)

The ML and BI phylogenetic reconstruction methods retrieved incongruent topologies for the cnuDNA dataset, therefore the topologies are presented separately (Fig. 4). The ML analysis supported three clades. The sister relationship between clade 1 and clade 2 had a bootstrap support of 98% (p*P* <0.95). With the exception of Cathedral Peak (a), all specimens from clade 1 were from localities occurring in the uMkomazi drainage system. Similarly, with the exception of Highmoore Nature Reserve, clade 2 included two sub-clades (2A and 2B) and was made up of specimens from the Tugela drainage system, with a bootstrap support of 86% (p*P* <0.95) (Fig. 4). Clade 2A was well-supported by ML (ML = 84%, p*P* <0.95), with Mahai, Gudufalls, Oliviershoek Pass, and Monks Cowl (1a) sister to an unsupported clade 2B, which included the rest of the Monks Cowl specimens (2a, 1b, and 2b), Cathedral Peak (a), Higmoore Nature Reserve, and Injisuthi (a). Injisuthi (a) was the only supported grouping within clade 2A (ML = 78%, p*P* <0.95). Injisuthi (b) was a clade on its own (ML = 100%, p*P* <0.95; clade 3, Fig. 4), corroborating the cmtDNA results (Fig. 3).

Conversely, the BI results were less resolved, only revealing that the Tugela and uMkomazi drainage systems formed a monophyletic clade (p*P* = 1.00; Fig. 4). According to the analysis convergence was reached because split frequencies remained below 0.01 and ESS values were >100. The analysis retrieved one supported grouping of the two drainage systems. Nonetheless, it was evident that, even without p*P* support, the uMkomazi and Tugela drainages formed two separate groups. Cathedral Peak (b) and Highmoore Nature Reserve (b) from the Tugela drainage system remained nested within the uMkomazi drainage specimens. Highmoore Farm clustered on its own, but within the grouping of uMkomazi specimens, as was observed from the cmtDNA (Fig. 3) and ML (nuDNA; Fig. 4). Within

clade support was only observed with the Injisuthi populations (Injisuthi (a): p*P* = 0.99; Injisuthi (b): p*P* = 1.00). Incidentally, the BI phylogenetic reconstructions showed an unsupported sister relationship between the two Injisuthi populations.

Combined cmtDNA and cnuDNA

The combined dataset (mt- and nuDNA), to a large extent, supported the cmtDNA topology (Fig. 5). While the ML and BI topologies were congruent, once again BI showed limited support for some clades that were supported by the ML analysis (Fig. 5). Notably, one of the differences between the cmtDNA and cnuDNA topologies (see Fig. 6 for comparison) is that Monks Cowl (b) formed a clade (ML = 99%, p*P* < 0.95; clade 1D), while Monks Cowl (a) locality grouped with the rest of the Tugela drainage localities (ML = 85%, p*P* <0.95; clade 2A, Fig. 5). Another, difference was that the two Injisuthi localities mixed, i.e. both specimens from Injisuthi (b) formed a basal clade to the species complex with one specimen from Injisuthi (a) (ML = 81%, p*P* <0.95; clade 4), while Cathedral Peak formed a separate clade with the other Injisuthi (a) specimen (ML = 89%, p*P* <0.95; clade 3). The addition of the nuDNA markers, which are slower evolving, reinforced the presence of multiple gene pools within the Cathedral Peak, Injisuthi, and Monks Cowl, and Highmoore localities.

SPECIES TREE, SPECIES DELIMITATION, AND DIVERGENCE TIME ESTIMATION

The species tree retrieved from *BEAST suggested eight unique lineages (Fig. 7), corroborating the combined dataset (Fig. 5). The species tree obtained from *BEAST with posterior probability 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 (a = *Potamonautes* sp. 5; b = *Potamonautes* sp. 1), Injisuthi (a = *Potamonautes* sp. nov.5; b = *Potamonautes* sp. 6), Monks Cowl (1a = *P. clarus*; b and 2a = *Potamonautes* sp. 4), and Highmoore Nature Reserve (a = *Potamonautes* sp. 4; b = *P. depressus*), Ndawana (a = *Potamonautes* sp. 3; b = *Potamonautes* sp. 2), Garden Castle Nature Reserve (a = *Potamonautes* sp. 1; b = *Potamonautes* sp. 3), Kamberg (a = *P. depressus*; b = *Potamonautes* sp. 5), and Lotheni (a = *P. depressus*; b = *Potamonautes* sp. 1), corroborating the phylogenetic reconstruction results. Thus, 32% of the 25 sampled localities harboured sympatric cryptic lineages. *Potamonautes* sp. 1 was made up of one locality from the Tugela drainage system (Cathedral Peak) and six localities from the uMkomazi drainage system (Fig.

7). *Potamonautes* sp. 2 and *Potamonautes* sp. 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), corroborating the parsimony haplotype network and all phylogenetic reconstruction topologies, except the nuDNA BI topology.

The species tree and divergence time tree topologies were near congruent. Divergence time estimations revealed that the *P. clarus* / *P. depressus* species complex diverged from its most recent common ancestor between mid- and late Miocene (12.1 Mya). Though ongoing, divergence within the species complex occurred between *c*. 10.3 Mya, where *Potamonautes* sp. 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. 2. Divergence between *Potamonautes* sp. 1 to *P. clarus* took place approximately 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. 5 / *Potamonautes* sp. 4 group 4.8 Mya (95% HPD: 2.1 – 8.1 Mya). The two clades comprising *Potamonautes* sp. 5 and *Potamonautes* sp. 4 separated 2.0 Mya (95% HPD: 0.7 – 4.0 Mya). The remaining four species (*Potamonautes* sp. 1 – *P. depressus*), split from each other 3.4 Mya (95% HPD: 1.5 – 5.8 Mya), separating *Potamonautes* sp. 1 from the other three lineages. *Potamonautes depressus* separated from the *Potamonautes* sp. 3 / *Potamonautes* sp. 2 group approximately 2.0 Mya (95% HPD: 0.7 – 4.0 Mya), and *Potamonautes* sp. 2 diverged from *Potamonautes* sp. 3 approximately 1.3 Mya (95% HPD: 0.4 – 2.6 Mya).

DISCUSSION

This study's novel approach in the delimitation of South African freshwater crab species has 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 other two are described as *P. clarus* and *P. depressus.* 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. 7). Concatenating the data of the six loci yielded seven (concatenated mtDNA) and eight (combined mt- and nuDNA) possible lineages, with some incongruencies observed between the gene trees (Figs. 3, 4, and 5) and the species tree (Fig.

7). Incongruencies between 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; Nordborg, 2001; Edwards, 2009; Knowles, 2009; Yang and Rannala, 2010; Leaché & Fujita, 2010; Carmago & Sites, 2013; Rannala & Yang, 2013). Moreover, unlike gene tree based species delimitations (or morphology and single locus-based delimitations), the Bayesian species delimitation method employs all loci (species tree) to lower the risk of inaccurate delimitations (Yang & Rannala, 2010).

Of the six loci used in the present study, PEPCK was found to be the least variable as opposed to the fastest evolving marker, COI. However, according to Tsang *et al*. (2008), protein-coding nuDNA markers such as PEPCK and NaK (Sodium-Potassium ATPase α-subunit) were ideal for phylogenetic delineation among decapods. While this may be true for 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 lineages. According to Yang and Rannala (2010), at least five loci are required for Bayesian species delimitation; therefore, the invariability of PEPCK did not influence the results, because mtDNA is more informative than nuDNA (Fig. 6).

Cryptic diversity within freshwater crabs is well documented (Daniels *et al*., 1998, 1999b; 2003; Jara *et al*., 2003; Jesse *et al*., 2010; Cumberlidge & 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 *Potamonautes*. This study supported the monophyly of *P. clarus*, which occurs in the northernmost bounds of the Tugela drainage system (Gouws *et al*., 2000; Daniels *et al*., 2003). Daniels *et al*. (2003) previously reported that the true *P. clarus* occurred in three localities in the northern parts of the Tugela drainage system. In the present study I found that the same populations (i.e. Gudufalls, Mahai, and Oliviershoek Pass) maintained their grouping with the addition of Monks Cowl (Fig. 7).

Potamonautes clarus was found to be sister to two cryptic sister lineages (*Potamonautes* sp. 4 and *Potamonautes* sp. 5, Fig. 7) comprising specimens from Monks Cowl and Highmoore Nature Reserve. *Potamonautes* sp. 4 is unique to the present study. I also identified two cryptic lineages within Highmoore Nature Reserve, one within *Potamonautes* sp. 4 and the other within *P. depressus* (Fig. 7). I recovered the same grouping between the sympatric lineage from Cathedral Peak and Injisuthi as Daniels *et al*. (2003); however this clade also included specimens from Kamberg (*Potamonautes* sp. 5, Fig. 7).

From the present study, it is apparent that Injisuthi comprises two sympatric lineages, one that was not identified by Daniels *et al.* (2003), thus novel to this present study (*Potamonautes* sp. 6, Fig. 7).

According to Daniels *et al*. (2003), *P. depressus*, was restricted to Lotheni and Kamberg. However, additional sampling has revealed that the species has a wider distribution, also occurring around Highmoore. This is supported by the shared haplotypes between Kamberg and Highmoore Nature Reserve (Fig. 2, haplotype 31). This finding also points to a sympatric occurrence of cryptic lineages in Kamberg. Therefore, because Daniels *et al*. (2003) sampled fewer localities, their cryptic lineage clusters may have been superficial.

Here, I expanded the number of occurrence localities of a lineage recognized by Daniels *et al*. (2003) as a sympatric lineage occurring in Cathedral Peak. Daniels *et al*. (2003) clustered this lineage with Doreen Falls (not sampled in this study). I identify this lineage as *Potamonautes* sp. 1 (Fig. 7). Together with Cathedral peak, Lotheni was the only other locality that also occurred with localities of *Potamonautes* sp. 1. This was consistent with the phylogenetic reconstruction results from the cmtDNA (Fig. 3) and combined cmt- and cnuDNA (Fig. 5), suggesting that the presence two sympatric lineages within Lotheni. Another locality that possesses sympatric lineages is Garden Castle Nature Reserve, which was found to occur within *Potamonautes* sp. 1 and *Potamonautes* sp. 3, which is another lineage unique to the present study (Fig. 7).

Notably, *Potamonautes* sp. 2 comprised specimens from Coleford, a locality that was also sampled by Gouws *et al*. (2000). Previously, Gouws *et al*. (2000) recognized specimens from this locality as *P. depressus* by assuming that all morphologically identical species occurring south of Kamberg (inclusive) were *P. depressus*. Besides, specimens from Ndawana (also found within *Potamonautes* sp. 3), all of the specimens in this lineage are unique to this study, i.e. *Potamonautes* sp. 2 is a novel lineage. Therefore, the taxonomy of the freshwater crabs that occur between Kamberg and Coleford needs revision because not all lineages that occur between and within these drainages are *P. depressus*. As such, *P. depressus* requires redescription and species boundaries need to be re-established. Thus, from the results, I deduce that *P. depressus* only occurs in four localities (Highmoore Farm and Nature Reserve, Kamberg, and Lotheni) and that the rest of the localities below Kamberg and Lotheni (localities 11 and 12, Fig. 1) harbour separate lineages that are yet to be described.

Therefore, I have recovered an additional three novel lineages (to Daniels' (2003) three lineages) along the Drakensberg Mountain range. Although freshwater crabs have

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recently been shown to harbour high cryptic diversity (Daniels *et al*., 2003; Jesse *et al*., 2010; Phiri & Daniels, 2014; Cumberlidge & Daniels, 2014), for southern African freshwater crabs, this level (32%) of sympatry has never been recorded. At present, I 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 cmtDNA and cnuDNA did not show this relationship (Fig. 3). 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. The divergence time results of between 10.3 and 0.11 Mya should be interpreted with caution because the high 95% HPD range is 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 & Millar, 1971; Dingle *et al*., 1983; Schmitz & Rooyani, 1987). 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 (Partridge & Maud, 2000). For other parts of South Africa, particularly the west, the Miocene / Pliocene is associated with intense upliftment episodes (Cowling *et al*., 2009), followed by periods of stasis and erosion (Partridge & Maud, 1987; Partridge, 1998; Partridge & Maud, 2000). In western South Africa, along the Cape Fold Mountain range, it is widely documented that this 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 (Daniels *et al*., 2004; Daniels *et al*., 2007; Cowling *et al*., 2008; Swart *et al*., 2009; Swartz *et al*., 2009; Linder *et al*., 2010; ; Stanley *et al*., 2011; Diedericks & Daniels, 2014). 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. According to Rivers-Moore *et al*. (2007), there are many isolated headlands as well as detached river valleys within the KwaZulu-Natal drainage network. 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 *et al*, 1992; Belliard *et al*., 1997; Cook *et al*., 2002; Gascon *et al*., 2002; Fraser & Keddy, 2005). Within KwaZulu-Natal, these

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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. I have recognized some lineages that have restricted distribution ranges. Here I confirmed that *P. clarus* is restricted to the northern bounds of the Tugela drainage system as previously recorded by Gouws *et al*. (2000) and Daniels *et al*. (2003). This area is maintained by the well-managed Ezemvelo KZN Wildlife conservancy and is regarded as one of the high priority freshwater conservation bioregions for conservation in KwaZulu-Natal (Rivers-Moore & Goodman, 2010). 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. 3 and *Potamonautes* sp. 4) 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 not well-looked after during my sampling efforts in September 2011 (pers. obs.). This could potentially pose a risk to the novel freshwater crab lineage (*Potamonautes* sp. 2) that is yet to be described. Although the species is present in 5 other localities, I am uncertain of how wide its distribution range might be, considering that Coleford was the southernmost locality that I 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 *et al*., 2011). In a detailed conservation

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plan by 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, they 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.

Conclusion

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, I 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 & Daniels, 2014). For example, in Phiri & Daniels (2014), I found no variation in gonopods 1 and 2 among three cryptic species recovered from the *P. perlatus* species complex. In spite of this, I am not suggesting the total disregard for morphology when delineating freshwater crab species. Instead, I suggest that species boundaries should be evaluated in relation to the morphology, genetic data, ecological boundaries (Padial *et al*., 2009) as well as the geological timescale. The morphological description of the 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, I focused on the high-lying areas (Drakensberg Mountain range) of the KwaZulu-Natal Province. As such, there remains some degree of uncertainty with regards to the level of cryptic diversity (at least for freshwater crabs) within the province. Although relative to other African countries, South Africa is the most comprehensively sampled country for freshwater crab diversity, the discovery of six undescribed freshwater crab lineages suggests that it is highly probable that continued sampling will retrieve more novel freshwater crab species, particularly throughout the rest of the KwaZulu-Natal Province in low-lying areas. In general, this study places emphasis on the need to revise the taxonomy of *Potamonautes*. 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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Table 1 Sampling localities (including geographical position) for the *Potamonautes clarus* / *P. depressus* species complex and the respective drainage systems where sampling was conducted. *N* = Sample size.

Drainage system	Collection locality (numbers are locality positions on map, Fig.1)	*N*	Latitude (S)	Longitude (E)
Tugela River				
	1 Oliviershoek Pass	5	-28.575833	29.053667
	2 Gudu Falls	5	-28.681278	28.941333
	3 Mahai	4	-28.689611	28.949389
	4 Cathedral Peak	5	-28.947944	29.483028
	5 Monk's Cowl	5	-29.044028	29.398944
	6 Monk's Cowl a	3	-29.038694	29.387556
	7 Injisuthi b	5	-29.103361	29.501139
	8 Injisuthi a	5	-29.103444	29.488389
	9 Highmoore Nature Reserve	5	-29.321028	29.618111
	10 Highmoore Farm	2	-29.328250	29.675806
uMkomazi River				
	11 Kamberg	5	-29.381083	29.660556
	12 Lotheni	5	-29.455556	29.742250
	13 Vergelegen Nature Reserve	5	-29.538111	29.451111
	14 Vergelegen	5	-29.575333	29.523028
	15 Sani Pass	5	-29.622583	29.392806
	16 Sani Pass Hotel	3	-29.655167	29.454250
	17 Cobham	5	-29.696194	29.401972
	18 Himeville	5	-29.722250	29.521222
	19 Lower Mzimkhulu	5	-29.030361	29.831222
	20 Garden Castle Nature Reserve	5	-29.756250	29.433111
	21 Lower Garden Castle	5	-29.745722	29.209361
	22 Bushman's Nek	5	-29.901917	29.296611
	23 Ndawana	5	-29.934472	29.373611
	24 Rougham	5	-29.891167	29.398250
	25 Coleford	5	-29.958861	29.474333
Total		117		

Table 2 The molecular markers and primer pairs used in this study with their respective polymerase chain reaction conditions. Temperatures in bold under PCR profile indicate the annealing temperatures. The final extension was at 72 °C for 10 minutes (7 min. for DecapANT). * Protein coding. ** Exon-priming, intron-crossing.

Molecular markers	Product size (pb)	PCR	Primer sequence (5'-3' direction)	PCR profile	Reference
Mitochondrial (mt)					
12S rRNA	353	12Sai 12Sb	AAACTAGGATTAGATACCCTATTAT GAGAGTGACGGGCGATGTGT	95 °C (5 min.), [95 °C (30 s), **50 °C** (40 s), 72 °C (1 min.)] x 34	Kocher *et al.*, 1989
16S rRNA	536	16Sa-1471 16Sb-1472	ACTTGATATATAATTAAAGGGCCG CTGGCGCCGCTCTGAACTCAAATC	95 °C (5 min.), [95 °C (30 s), **50 °C** (40 s), 72 °C (1 min.)] x 34	Palumbi *et al.*, 1991
*Cytochrome oxidase subunit I	625	LCOI-1490 HCOI-2198	GGTCAACAAATCATAAAGATATTG TAAACTTCAGGGTGACCAAAAAATCA	94 °C (4 min.), [94 °C (30 s), **42 °C** (40 s), 72 °C (45 s)] x 36	Folmer *et al.*, 1994
Nuclear (nu)					
28S rRNA	620	28Sa-modified 28Sb	GACCCGTCTTGAARCACGGA TCGGAAGGAACCAGCTAC	94 °C (4 min.), [94 °C (45 s), **50 °C** (1 min.), 72 °C (1 min.)] x 40	Jesse *et al.*, 2010
**Adenine nucleotide transporter	455	DecapANT-F DecapANT-R	CCTCTTGAYTTCGCKCGAAC TCATCATGCGCCTACGCAC	94 °C (3 min.), [94 °C (30 s), **60 °C** (30 s), 72 °C (30 s)] x 35	Teske & Beheregaray, 2009; Teske *et al.*, 2009
*Phosphoenolpyruvate carboxykinase	493	PEPCK-for PEPCK-rev	GTAGGTGACGACATTGCYTGGATGAA GAACCAGTTGACGTGGAAGATC	94 °C (3 min.), [94 °C (30 s), **60 °C** (45 s), 72 °C (1 min. 30 s)] x 35	Tsang *et al.*, 2008

Table 3 Model parameters for each locus used in this study, where (a) represents the best-fit model for the Bayesian analyses as obtained in jModelTest v. 2.1.3 and (b) is nucleotide substitution models as obtained in MEGA5 v. 2.2 (* for Maximum Likelihood analyses only, obtained from MEGA 5 v. 2.2.

Gene fragment	Model	Base pair frequencies (%)	Gamma (G) distribution parameter	Proportion invariable (I) sites
12S rRNA	(a) TPM3uf + G (nst = 6; -lnL = 879.95; AIC = 1975.91)	A = 36.98 C = 8.63 G = 18.35 T = 36.04	0.013	N/A
	(b) T92 + I; -ln L = 899.90; AIC = 2009.10)	A = 36.12 C = 13.88 G = 13.88 T = 36.12	N/A	0.779
16S rRNA	(a) TPM2uf + G (nst = 6; -lnL = 1722.17; AIC = 3660.35)	A = 37.32 C = 9.50 G = 16.39 T = 36.79	0.208	N/A
	(b) TN93 + I; -lnL = 1741.12; AIC = 3697.10)	A = 37.70 C = 9.90 G = 17.0 T = 35.4	N/A	0.660
COI	(a) TIM2 + G + I (nst = 6; -lnL = 2446.72; AIC = 5113.44)	A = 28.81 C = 19.05 G = 15.30	1.095	0.603

Table 3 continued

		T = 36.84		
	(b) TN93 + G + I (-lnL = 2463.18; AIC = 5143.11)	A = 29.30 C = 19.30 G = 15.80 T = 35.60	0.720	0.570
28S rRNA	(a) TIM3 + G (nst = 6; -lnL = 1263.51; AIC = 2745.01)	A = 14.95 C = 31.15 G = 34.89 T = 19.02	0.157	N/A
	(b) T92 (-lnL = 1286.30; AIC = 2779.40)	A = 16.89 C = 33.11 G = 33.11 T = 16.89	N/A	N/A
DecapANT	(a) TPM2uf + G (nst = 6; -lnL = 844.78; AIC = 1903.556)	A = 23.34 C = 20.82 G = 26.24 T = 29.60	N/A	N/A
	(b) K2 (-lnL = 849.85; AICc = 1904.80)	A = 25.00 C = 25.00 G = 25.00 T = 25.00	N/A	N/A
PEPCK	(a) HKY + G + I (nst = 2; -lnL = 1059.05; AIC = 2334.09)	A = 19.51 C = 35.73	0.396	0.824

Table 3 continued

		G = 27.99 T = 16.77		
	(b) T92 + G + I (-lnL = 1261.09; AIC = 2733.11)	A = 18.20 C = 31.80 G = 31.80 T = 18.20	3.590	0.930
*cmtDNA	T92 + G (-lnL = 5220.92; AIC = 10642.13)	A =34.67 C = 15.33 G = 15.33 T = 34.67	0.129	N/A
*cnuDNA	T92 + G + I (-lnL = 3677.55; AIC = 7565.40)	A = 19.80 C = 30.20 G = 30.20 T = 19.80	0.430	0.830
*cmtDNA + cnuDNA	T92 + G + I (-lnL = 10430.85; AIC = 21071.84)	A = 27.30 C = 22.70 G = 22.70 T = 27.30	0.760	0.750

Table 4 Haplotype frequencies by locality. Localities: 1 = Bushamans Nek, 2 = Ndawana, 3 = Cobham, 4 = Coleford, 5 = Himeville, 6 = Rougham, 7 = Garden Castle Nature Reserve, 8 = Sani Pass, 9 = Lower Garden Castle, 10 = Sani Pass Hotel, 11 = Vergelegen, 12 = Vergelegen Nature Reserve, 13 = Lotheni, 14 = Cathedral Peak, 15 = Lower Mzimkhulu, 16 = Highmoore Farm, 17 = Kamberg, 18 = Highmoore Nature Reserve, 19 = Gudufalls, 20 = Mahai, 21 = Oliviershoek Pass, 22 = Monks cowl(a), 23 = Monks cowl(b), 24 = Injisuthi(b), 25 = Injisuthi(a)

Locality	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Haplotype																									
H1	2	4	2	3	2																				
H2				1		4																			
H3	1				1																				
H4				1																					
H5	1				1																				
H6			1																						
H7			1																						
H8						1																			
H9			1																						
H10					1																				
H11	1																								
H12					1		1	2	3	2	3	3													
H13							1					1													
H14							1		1		1	1	1												
H15								2																	
H16								1																	
H17													1												
H18													1												
H19														1											

Table 4 continued

H20								1		1		1										
H21							1															
H22	1																					
H23														2								
H24														1								
H25														1								
H26						2								1								
H27															1							
H28																1						
H29																	1					
H30															1							
H31												1					3					
H32																1						
H33																1						
H34																1						
H35																		1				
H36																		2				
H37																			2			
H38																			1			
H39																				2		
H40																				2		
H41																				1		
H42																					1	
H43																					1	
H44																					1	2

Table 4 continued

H45										2		
H46										1		
H47											1	
H48											3	
H49											1	
H50												2
H51												2
H52				1								
H53	4					1						
H54												
H55					1							

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Table 5 Uncorrected between clade (Fig. 3) sequence "p" distances.

Clade	Clade	"p" distance (%)
Clade 1a	Clade 1c	4.08%
Clade 1a	Clade 4	7.79%
Clade 1a	Clade 2b	9.47%
Clade 1a	Clade 2a	9.94%
Clade 1a	Clade 3	15.14%
Clade 1b	Clade 1a	4.33%
Clade 1b	Clade 1c	4.56%
Clade 1b	Clade 4	9.19%
Clade 1b	Clade 2b	9.80%
Clade 1b	Clade 2a	10.34%
Clade 1b	Clade 3	15.21%
Clade 1c	Clade 4	7.83%
Clade 1c	Clade 2a	9.30%
Clade 1c	Clade 3	14.70%
Clade 2b	Clade 2a	6.85%
Clade 2b	Clade 4	8.36%
Clade 2b	Clade 1c	9.07%
Clade 2b	Clade 3	13.80%
Clade 3	Clade 4	13.50%
Clade 3	Clade 2a	14.50%
Clade 4	Clade 2a	8.39%

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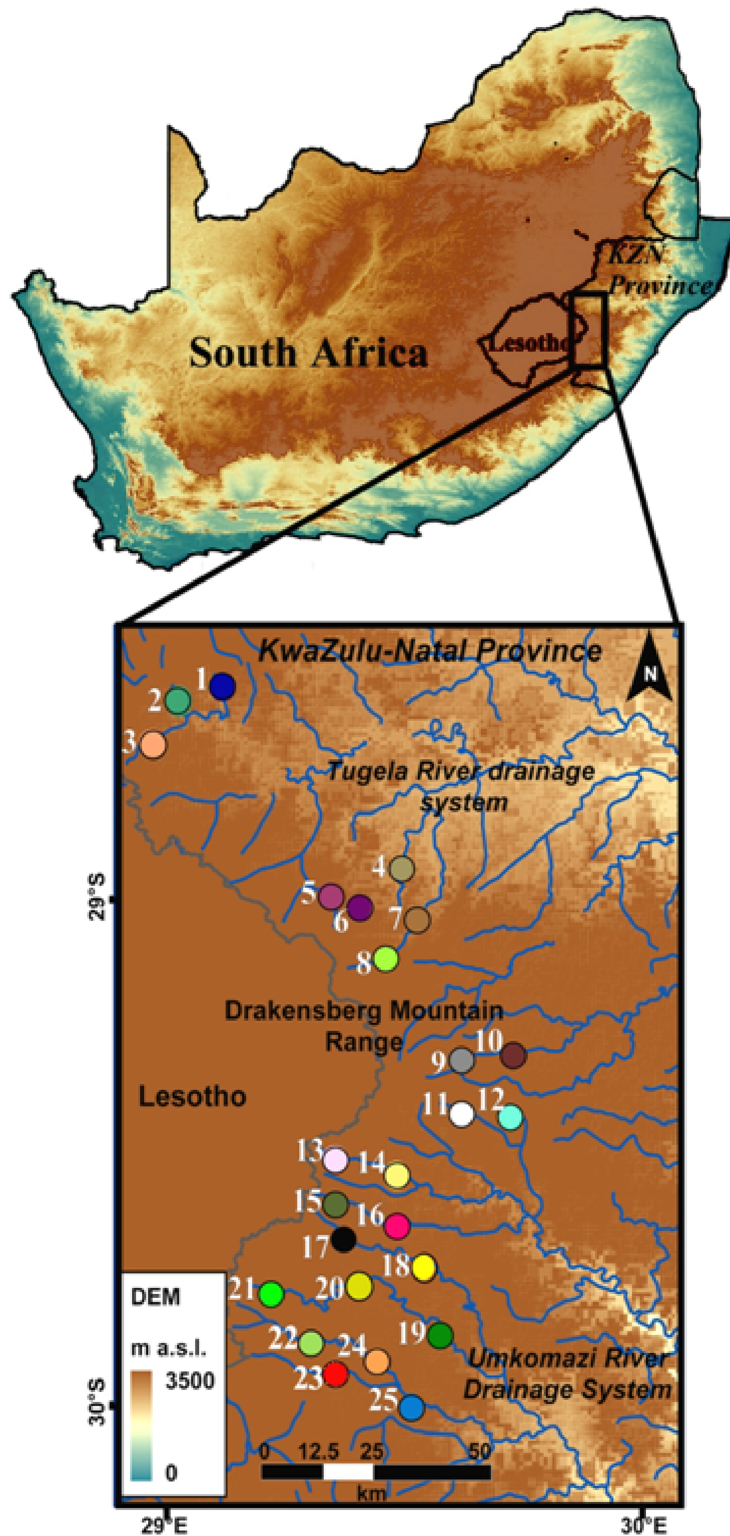

Figure 1 A sampling locality map showing the 25 localities where specimens of the *Potamonautes clarus* / *P. depressus* species complex were sampled. Each locality was designated a unique colour which corresponds with the haplotype network (Fig. 2).

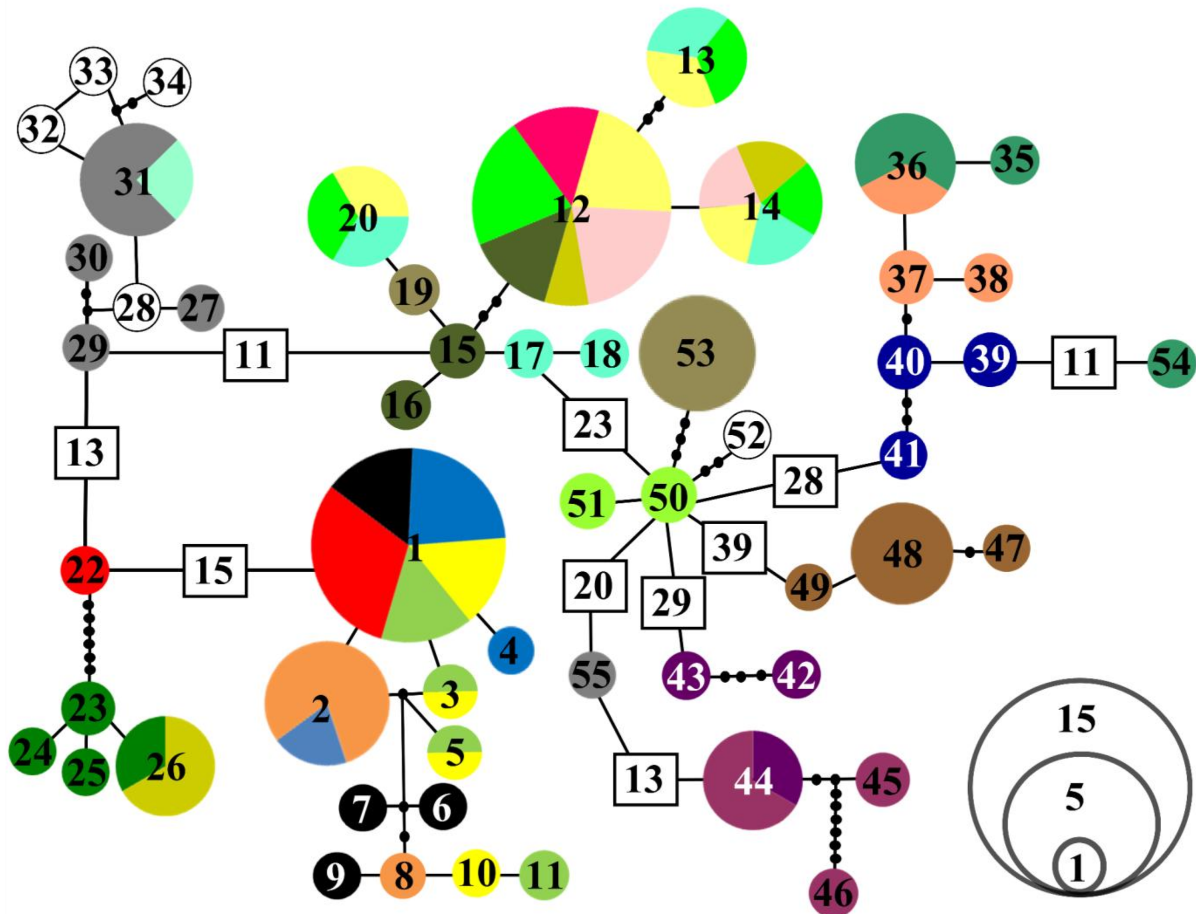

Figure 2 A 95% parsimony haplotype network for the *Potamonautes clarus* / *P. depressus* species complex using the COI gene. The small solid black circles represent the mutational steps or missing haplotypes within the connection limit of ten as shown by TCS, while the squares represent the connection distances (number of mutational steps) between haplotype groups; constructed in HapStar from the minimum spanning tree. The haplotype colours in pie charts correspond to the locality colours in Fig. 1 (values in the circles are the haplotype number).

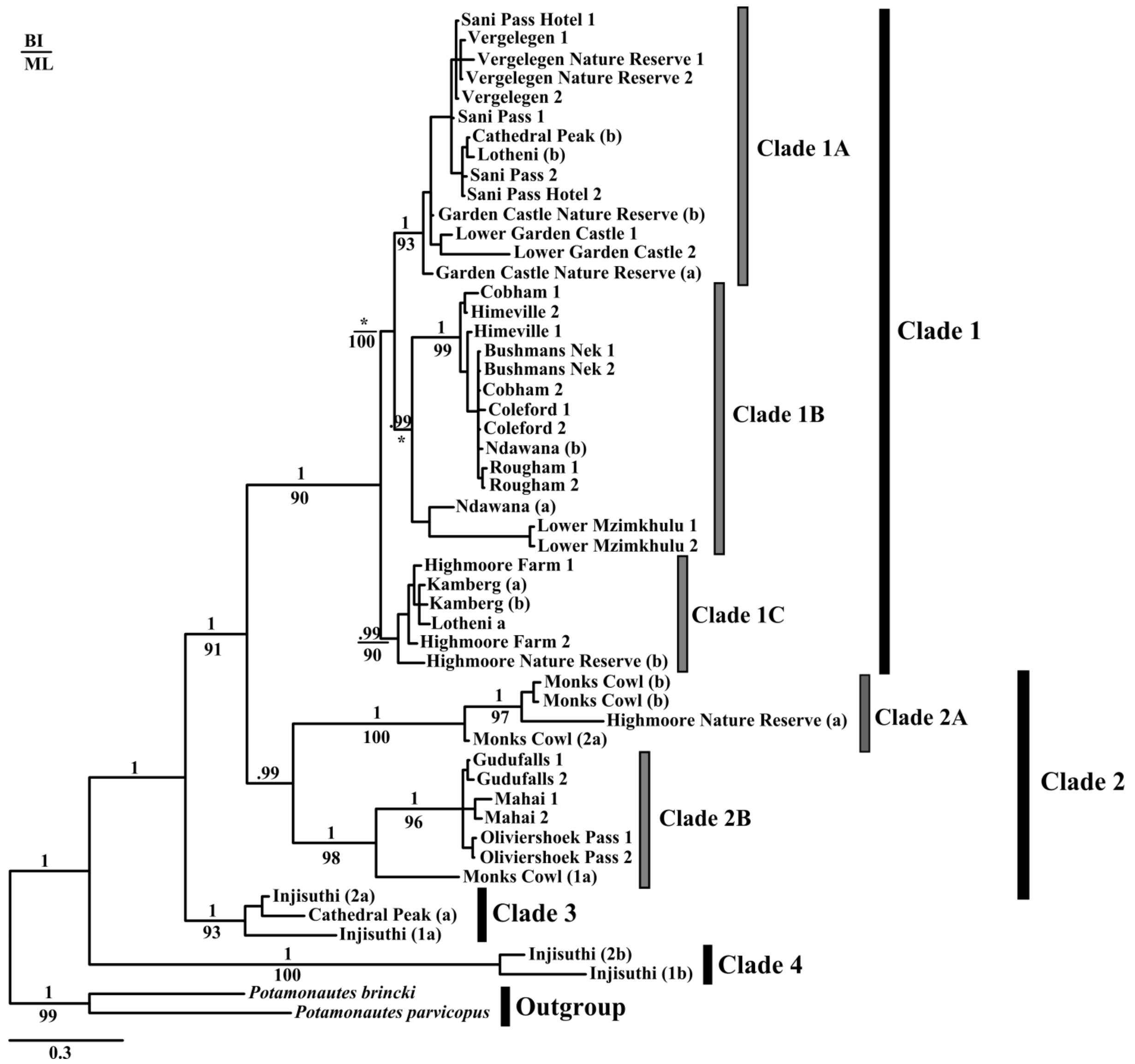

Figure 3 The concatenated mtDNA (12S rRNA, 16S rRNA, and COI) phylogram obtained from the ML analysis. Values above branches indicate BI posterior probability support, and values below branches are representative of ML bootstrap support. An * indicates no support.

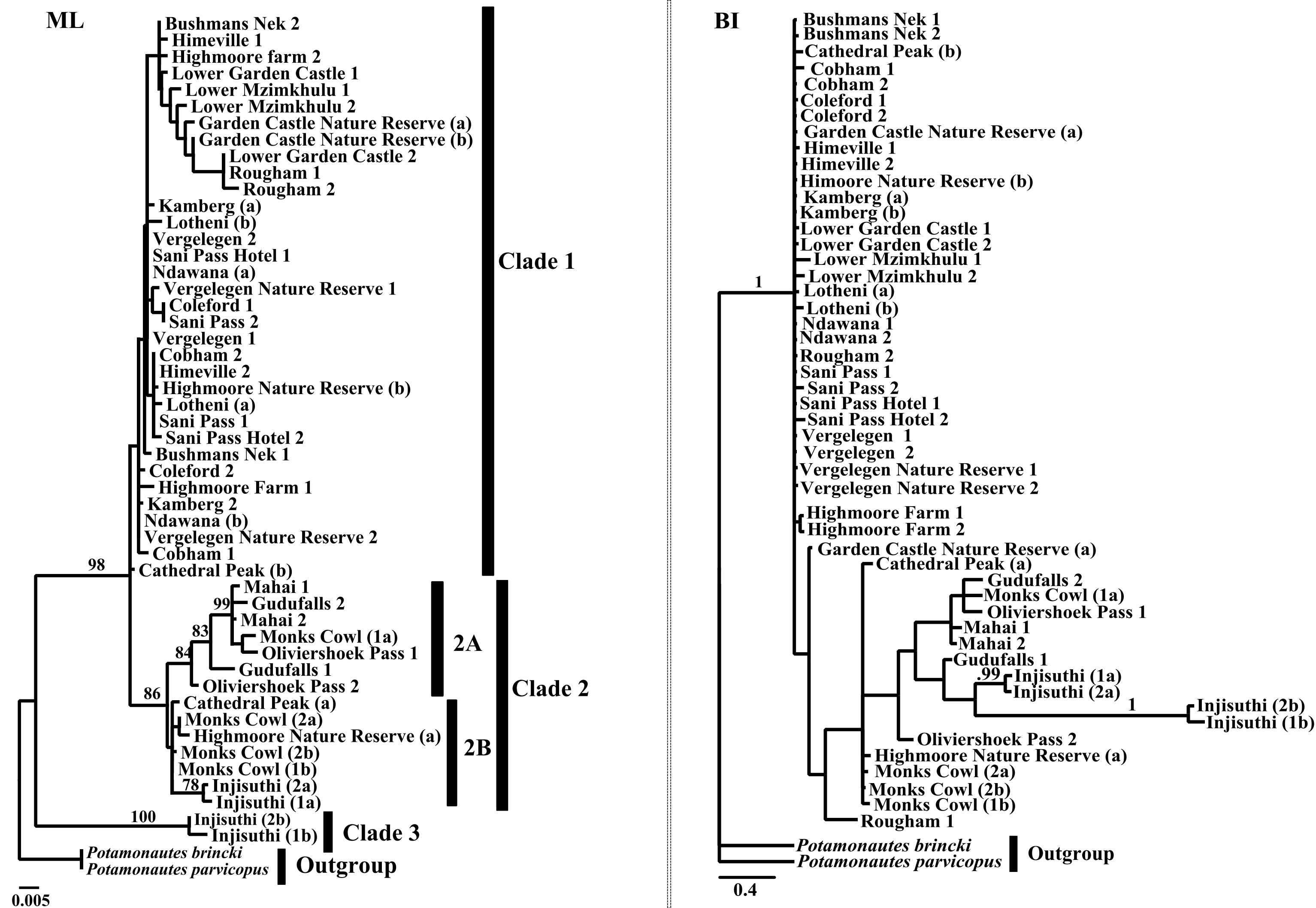

Figure 4 An ML phylogram (on the left) and BI consensus tree (on the right) of the concatenated nuDNA (28S rRNA, DecapANT, and PEPCK), with values above branches indicating ML bootstrap and BI p*P* respectively. Only values that indicated support (i.e. ML bootstrap ≥75% and BI p*P* ≥0.95).

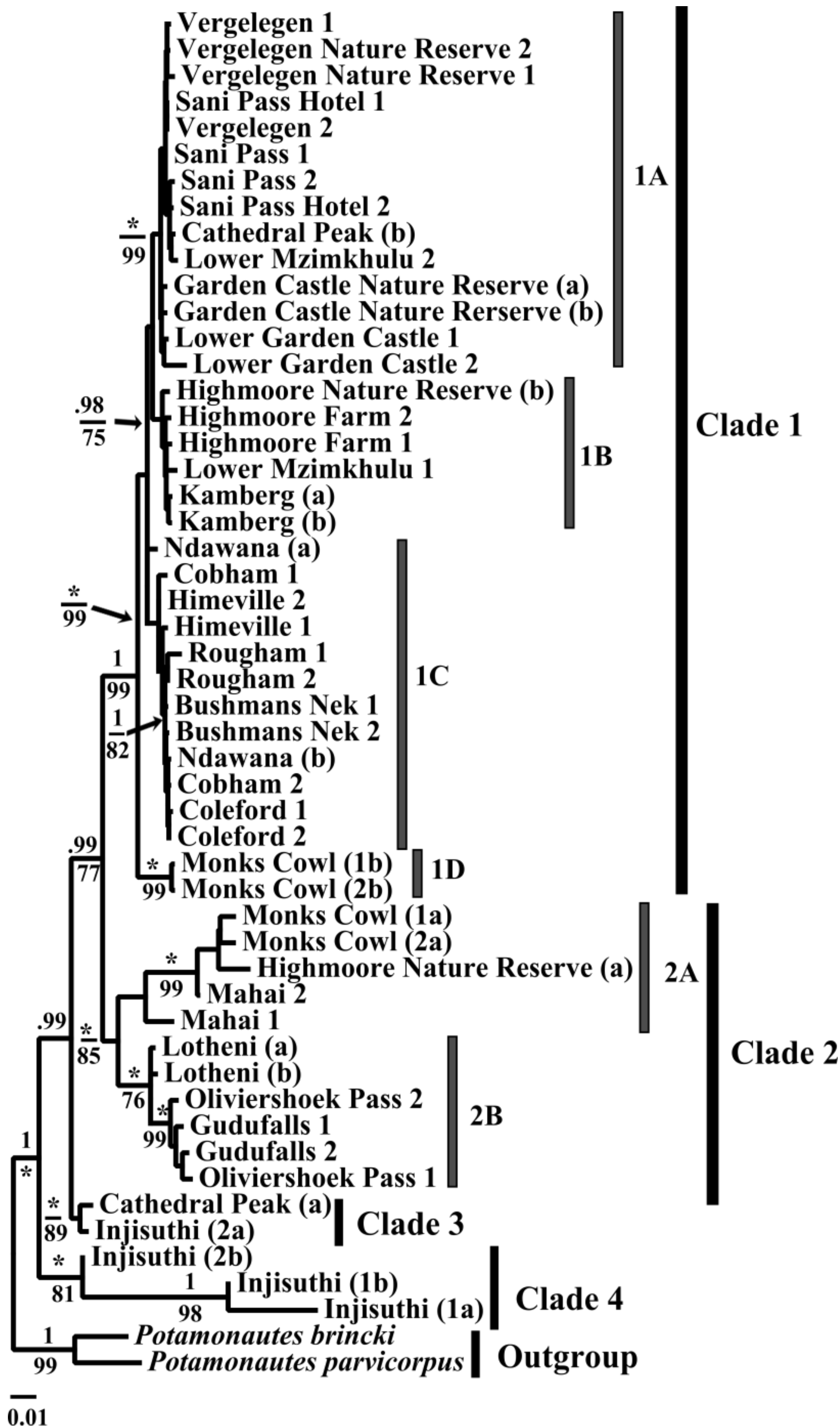

Figure 5 An ML phylogram of the concatenated mt- and nuDNA dataset (12S rRNA + 16S rRNA + COI + 28S rRNA + DecapANT + PEPCK). Values above branches indicate BI posterior probability support (p$P \geq 0.95$), and values below branches are representative of ML bootstrap support ($\geq$75%). An * indicates no support.

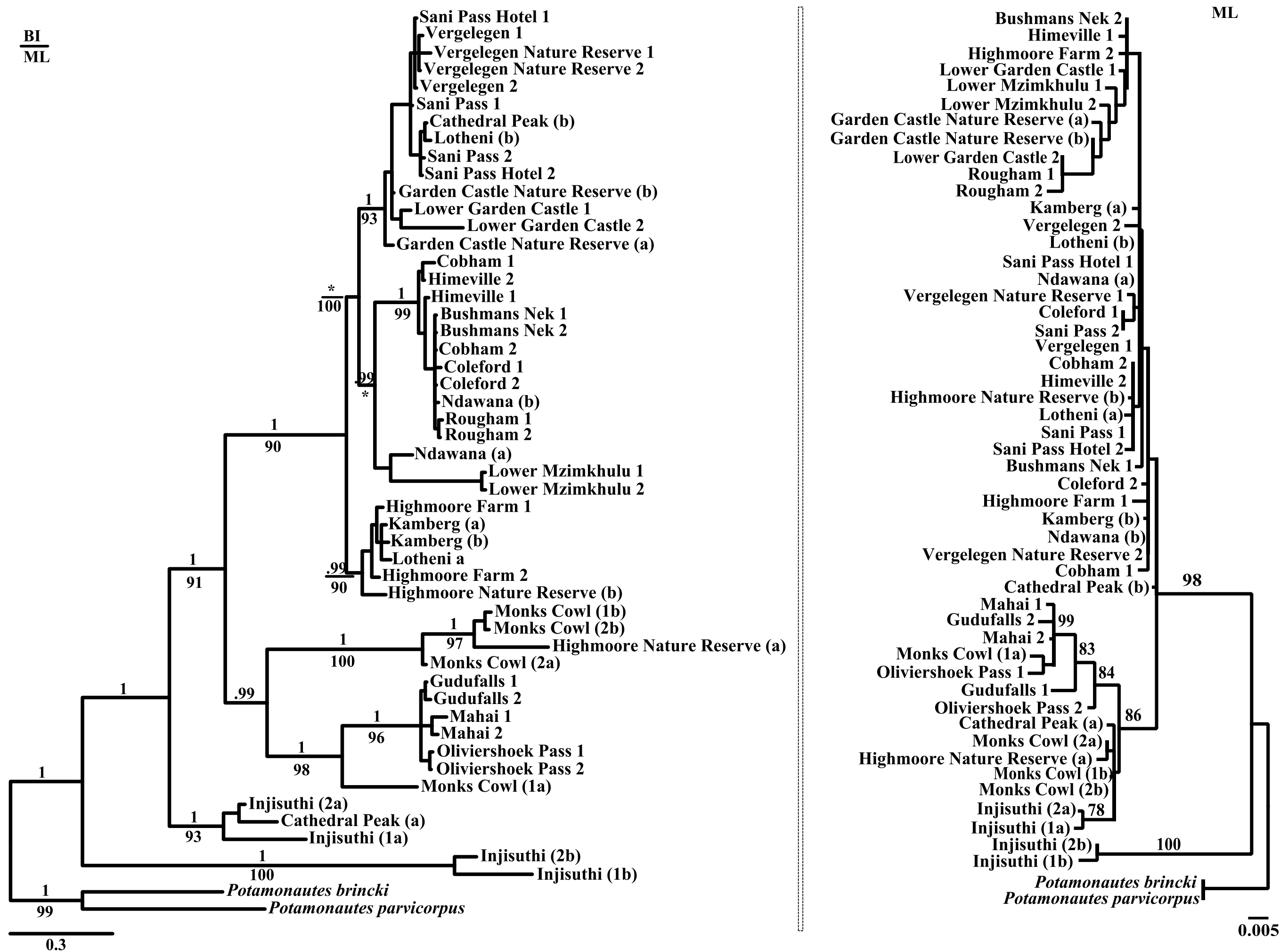

Figure 6 A comparison between the cmtDNA (left) and nuDNA (right) topologies. For the cmtDNA topology, values above branches indicate BI posterior probability support (p$P \geq 0.95$), and values below branches are representative of ML bootstrap support (≥75%). Only the ML support is indicated for the cnuDNA topoplogy (see Fig. 4). An * indicates no support.

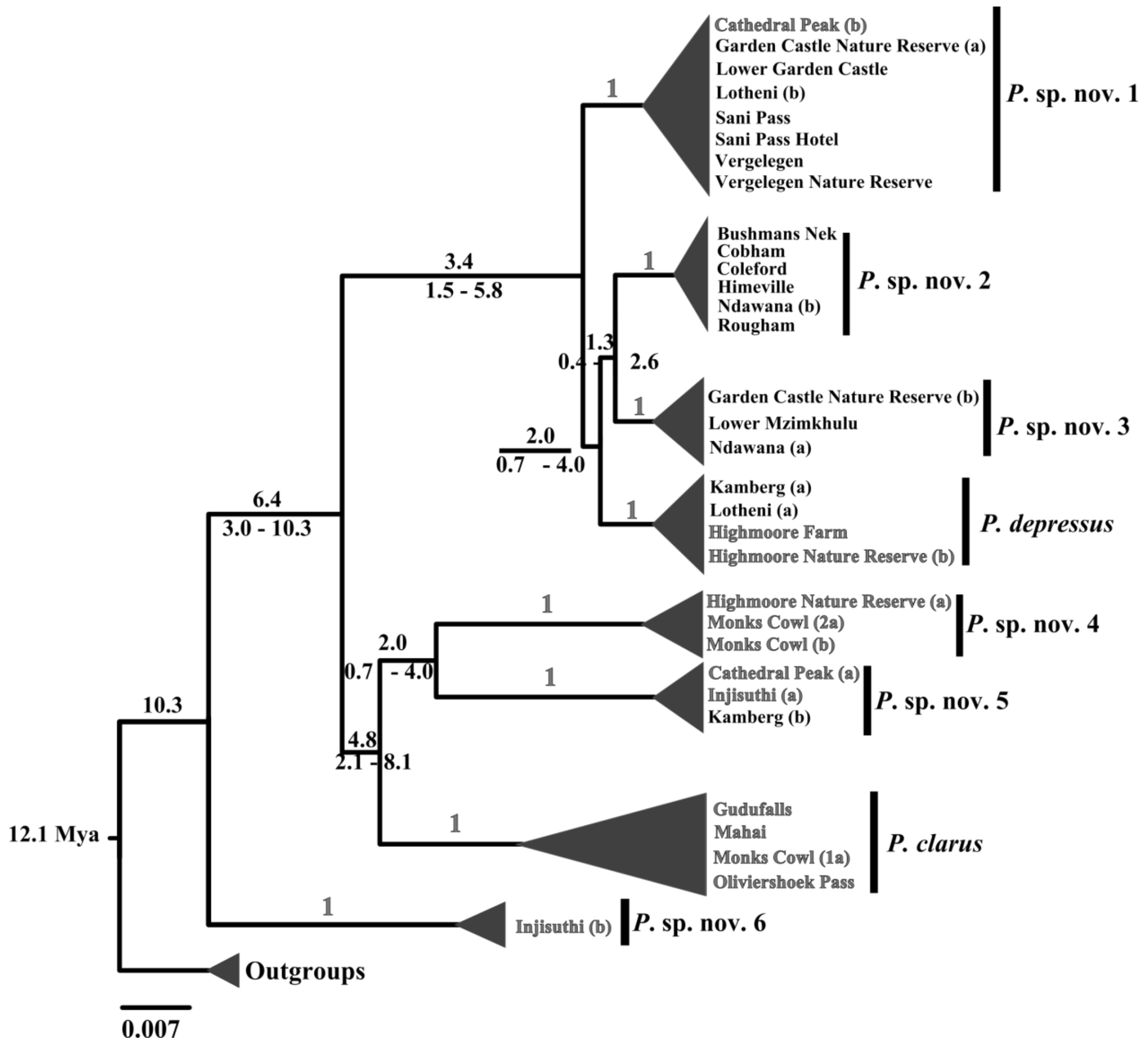

Figure 7 The species tree as given by *BEAST with posterior probability support of 1 (grey values) above the branches. The bold locality names represent specimens from the uMkomazi drainage while the localities in grey are representative of the Tugela drainage. On the outher branches, the black (bold) values show divergence times of the delimited lineages (above the branches) and the 95% highest posterior density (HPD) values for each divergence date. Note: this tree is not the divergence time tree, i.e. the divergence time for each lineage is merely plotted at the corresponding branches.

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CHAPTER 4

HIDDEN IN THE HIGHLANDS: THE DESCRIPTION AND PHYLOGENETIC POSITION OF A NOVEL ENDEMIC FRESHWATER CRAB SPECIES (POTAMONAUTIDAE: *POTAMONAUTES*) FROM ZIMBABWE [*]

ABSTRACT

A recent sampling endeavour of freshwater crabs along the high-lying streams of the Nyanga mountain range in Mutare (Eastern Highlands, Zimbabwe) yielded a morphologically distinct, as yet undescribed species. The novel Zimbabwean species is compared to the 16 described species from southern Africa based on mtDNA sequence data derived from three partial gene sequences (12S rRNA, 16S rRNA and COI). The new Zimbabwean species was found to be a sister taxon to *Potamonautes mulanjeensis*. These two species are morphologically and genetically easily differentiated. The new species is described as *Potamonautes mutareensis* sp. nov. and is compared morphologically to the known freshwater crab species of southern Africa. A dichotomous key to the four described freshwater crab species that occur in Zimbabwe is also provided. The results suggest that species diversity and endemism of freshwater decapods and other habitat specialists is likely to be high in unsampled mountainous regions.

INTRODUCTION

Freshwater crabs are among the largest detritivores found in the freshwater ecosystems of tropical regions where they play a central role in ecological processes (Dobson *et al.*, 2002, 2007). They reduce leaf litter particle size, which is important for microbial activity, thereby providing nutrition for filter-feeding aquatic fauna (Hill & O'Keeffe, 1992; Dobson *et al.*, 2002, 2007). Together with their ubiquity and high biomass in inland aquatic ecosystems, the role they fulfil renders them important contributors to the dynamics of nutrient recycling in river systems (Hill & O'Keeffe, 1992; Dobson *et al.*, 2002). Furthermore, because of their vulnerability to disturbances such as pollution, freshwater crabs have also been used as indicators for freshwater quality (Steenkamp *et al.*, 1993; Thawley *et al.*, 2004). Freshwater crabs are also important for supporting small commercial fisheries, with some species serving

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as a food source for a variety of commercially important fish species (Sachs & Cumberlidge, 1991; Cumberlidge *et al*., 1999; Cumberlidge & Daniels, 2008; Cumberlidge *et al*., 2009).

The Afrotropical region's freshwater crab fauna (including sub-Saharan continental Africa, Socotra, the Seychelles and Madagascar) represents approximately 10% of the global species diversity of freshwater crabs (Cumberlidge *et al*., 2008; Yeo *et al*., 2008). However, this is likely to be a significant underestimate because sampling and systematic studies in the majority of sub-Saharan African countries have not occurred for several decades (Cumberlidge & Daniels, 2008; Cumberlidge *et al*., 2009; Daniels & Bayliss 2012), hampering true estimation of the taxonomic diversity of freshwater crabs in the region.

Potamonautes MacLeay, 1838 (Potamonautidae) is the most widespread and diverse freshwater crab genus among the seven genera occurring in sub-Saharan Africa. *Potamonautes* occurs from the Nile valley in Egypt to South Africa and from Senegal in West Africa to the Horn of Africa in the east of the continent (Cumberlidge, 1999). The genus is absent from the Sahara desert and parts of north-west Africa, which biogeographically falls within the Palaearctic region (Cumberlidge, 1999). At present, there are an estimated 79 described potamonautid freshwater crab species, with several novel species being described annually (Cumberlidge & Clark, 2010, 2012; Daniels & Bayliss, 2012). Several novel freshwater crab species have in recent years been described from the sub-Saharan African region, including South Africa (Stewart & Cook 1998; Cumberlidge *et al*., 2002, 2009; Cumberlidge & Clark, 2010, 2012; Daniels & Bayliss, 2012). However, records of freshwater crab diversity in countries neighbouring South Africa, such as Mozambique and Zimbabwe, are limited as no recent systematic surveys of inland aquatic bodies (rivers, streams and lakes) have been conducted due to civil wars or political unrest. Hence, freshwater crab diversity in Mozambique and Zimbabwe has remained poorly explored.

However, recently, two new freshwater species were described from Mozambique and Malawi (Daniels & Bayliss, 2012). It was hypothesised that high-lying mountainous inland areas may be harbouring undescribed, often endemic species, and should be targeted in future studies (Daniels & Bayliss, 2012). The freshwater crab fauna of neighbouring Zimbabwe appears species poor and all three documented species – *Potamonautes bayonianus* Britto-Capello, 1864, *P. obesus* A. Milne-Edwards, 1868 and *P. unispinus* Stewart & Cook, 1998 – are widely distributed in southern Africa (Cumberlidge & Daniels, 2008; Darwall *et al*., 2009). Nevertheless, the apparent absence of endemic freshwater crab species might simply reflect a paucity in recent collection efforts in the country, considering the recent description of novel species from Malawi and Mozambique (Daniels & Bayliss, 2012).

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Consequently, the first author undertook a sampling trip to Zimbabwe to collect freshwater crabs in the Eastern Highlands. The Eastern Highlands is a narrow (*c.* 450 km long north–south) mountain belt, occurring along the eastern Zimbabwe–western Mozambique border. This mountain range is distinguishable by the high-elevation rim that encompasses the Nyanga (north) and Chimanimani (south) mountain ranges. The highest peak (2592 m above sea level (a.s.l.)) is found on Mount Nyangani (Moore, 1998). During the systematic survey of the drainages on the Zimbabwean portion of the Eastern Highlands, a freshwater crab species morphologically distinct from the three known species that occur in the country (and from all the described southern African species) was collected. Upon return to the laboratory, the species was subjected to morphological examination and mtDNA sequencing. This study formally describes the novel species and compares it to the known freshwater crab species of the region. A key to the freshwater crabs of Zimbabwe is provided.

MATERIALS AND METHODS

SAMPLE COLLECTION

Freshwater crab specimens were collected from five localities in the tributaries of the Save River on the Nyanga mountain range in Mutare (Eastern Highlands, Zimbabwe; Fig. 1). Crabs were collected by hand or baited with ox-heart lines, after which they were killed by freezing for 24 h before morphological character measurements and tissue extraction. One pereopod (walking leg) was broken off each specimen and preserved in 95% ethanol.

DNA EXTRACTION, PCR, AND SEQUENCING

DNA was extracted from muscle tissue of each pereopod using the NucleoSpin Tissue extraction kit (Machery-Nagel, Germany) following the manufacturer's protocol. The extracted DNA was stored at –20°C until required for polymerase chain reaction (PCR). The DNA was diluted 1 μL : 19 μL with millipore water. I amplified three mitochondrial partial gene fragments: 12S rRNA (12Sai 5'-AAA CTA GGA TTA GAT ACC CTA TTA T-3' and 12Sb 5'-GAG AGT GAC GGG CGA TGT GT-3') (Kocher *et al.,* 1989); 16S rRNA (16Sa 5'-ACT TGA TAT ATA ATT AAA GGG CCG-3' and 16Sb 5'-CTG GCG CCG CTC TGA ACT CAA ATC-3') (Palumbi *et al.,* 1991); and cytochrome oxidase I (COI) (LCOI-1490 5'-GGT CAA CAAA TCA TAAA GAT ATTG-3' and HCOI-2198 5'-TAAA CTT CAG GGT GAC CAAA AAA TCA-3') (Folmer *et al.,* 1994). Mitochondrial genetic markers have extensively been applied in crustacean phylogenetic studies, and specifically among the

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decapods, where they have proven useful in recovering phylogenetic relationships at various taxonomic levels (Daniels *et al.,* 2002, 2006*a*, 2006*b*; Shih *et al.,* 2006). Specifically, these three markers (12S rRNA, 16S rRNA and COI) were used in a recent study by Daniels & Bayliss (2012), where new freshwater crab species were identified in Malawi and Mozambique. The latter country borders Zimbabwe on the Eastern Highlands.

Standard PCR conditions and protocols were followed (see Daniels *et al.,* 2006*b*). Polymerase chain reaction products were electrophoresed for four hours in a 1% agarose gel containing ethidium bromide. The DNA fragments, cut out from the agarose gel, were then purified using the BioFlux gel purification kit (Bioer Technology Co., Ltd). The purified PCR products were sent to Macrogen Europe (Amsterdam, The Netherlands, www.macrogen.com) for sequencing.

PHYLOGENETIC ANALYSES

To validate the phylogenetic placement of the specimens collected in Zimbabwe, I used sequences of the 16 described southern African (Daniels & Bayliss, 2012) and two eastern African freshwater crab species. *Potamonemus sachsi* Cumberlidge & Clark, 1993 *Sudanonautes aubryi* H. Milne Edwards, 1853 and *Liberonautes rubrigimanus* Cumberlidge & Sachs, 1989 were used as outgroups (Daniels & Bayliss, 2012).

Datasets from all three loci were concatenated (1322 bp) for analysis because they are linked in the mitochondria, and are therefore expected to share similar evolutionary histories. I used maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) to construct phylogenetic trees. Maximum parsimony and ML were executed in MEGA5 v. 2.2 (Tamura *et al.,* 2011). For the MP analyses, the evolutionary histories were estimated by constructing a bootstrap consensus tree inferred from 5 x 10^3 replicates; branches of partitions reproduced in less than 50% of bootstrap replicates were collapsed. Both the ML and MP trees were obtained using the Close-Neighbour-Interchange algorithm (Nei & Kumar, 2000) and initial trees were obtained with the random addition of sequences. For ML, bootstrap consensus trees were inferred from 5 x 10^3 replicates that were taken to represent the evolutionary history of the taxa. The Tamura 3-parameter evolutionary model with gamma-distributed rate heterogeneity (T92 + G; obtained in MEGA5 v. 2.2) (Nei & Kumar, 2000; Tamura *et al.,* 2011) was used for the concatenated dataset in the ML analysis. For both MP and ML, nodal support was regarded as well resolved where bootstrap values ≥75%. Uncorrected (‘p’) distances were calculated for the COI locus using MEGA v. 2.2.

For the BI analysis, I first obtained the best-fit substitution models in jModelTest v. 2.1.3 (Posada, 2008). The best-fit maximum likelihood scores were chosen using Akaike's information criterion (AIC) (Akaike, 1973) to separate complex models, and reduce the number of parameters that did not contribute to describing the data (Burnham & Anderson, 2002; Nylander *et al.,* 2004). BI consensus trees were constructed in a partitioned analysis using the substitution model for each locus in MrBayes v. 3.2.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Four Markov chain Monte Carlo (MCMC) simulations were run for 2.5×10^6 generations with each chain starting from a random tree and chains were sampled every 2500th generation. A 50% majority rule tree was retained after 25% of the trees were discarded as burn-in. Consensus trees were viewed and edited in FigTree v. 1.4 (Drummond *et al.*, 2009). Branches with posterior probabilities (p*P*) ≥0.95 were regarded as well supported.

MORPHOLOGY

Specimens were sexed as either male (n = 14) or female (n = 12). One male holotype specimen was selected. Samples (including one holotype) were deposited at the South African Museum of Natural History (SAM), Iziko Museums of Cape Town. Morphological measurements were taken using digital vernier callipers. The characters that were measured (carapace and pereopods) are as follows (Table 1): carapace length measured along the medial line, CL; carapace width at the widest point, CWW; width of the posterior margin of the carapace, CWP; frontal width measured between the medial margin of the orbits, FW; distance between the postfrontal crest and the anterior margin of the carapace, PFCD; carapace height or depth, CH; major cheliped propodus length, MCPL; major cheliped propodus length, MCPH; length of the propodus of pereiopod 2, P2PL; width of the merus of pereiopod 2, P2MW; length of the propodus of pereiopod 5, P5PL; width of the propodus of pereiopod 5, P5PW (Sternberg *et al.,* 1999; Daniels *et al.,* 2002; Sternberg & Cumberlidge, 2003; Daniels & Bayliss, 2012). The holotype was photographed using a Nikon D300S digital camera (Nikon Corporation, Chiyoda, Tokyo, Japan), while gonopods 1 and 2 were photographed with a Leica MZ 16A digital camera attached to a Leica EC 3X stereomicroscope (Leica Microsystems, Heerbrugg, Switzerland).

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RESULTS

The combined dataset yielded 1322 base pairs (318 for 12S rRNA, 435 for 16S rRNA and 569 for COI). The sequences resulting from this present study were deposited in GenBank (Accession numbers: 12S rRNA KC768228-KC768255; 16S rRNA KC768256- KC768282; and COI KC768283-KC768307). The AIC substitution models for the three loci that were utilised in the Bayesian inference analyses were: TPM1uf + G (–ln L = 2145.19; AIC = 4618.39) for 12S rRNA; TrN + I + G (–ln L = 4607.8532; AIC = 9545.70) for 16S rRNA; and TIM3 + G (–ln L = 5678.40; AIC = 11686.79) for COI (see Table 2 for detailed information regarding each model).

All three phylogenetic reconstruction methods recovered congruent tree topologies. The clade comprising the undescribed specimens collected in Zimbabwe was statistically well supported (MP = 77%, ML = 91%, p*P* = 1.00; Fig. 2). The undescribed specimens were found to be a sister taxon to the recently described *Potamonautes mulanjeensis* Daniels & Bayliss, 2012. The latter two species were found to be sister to a clade comprising all the large-bodied riverine freshwater crab species from South Africa, while all the small-bodied mountain-living southern African freshwater crab species formed a group. In addition, all the remaining east African species formed a well-supported basal clade.

The minimum uncorrected sequence divergence for the COI locus between the undescribed Zimbabwean specimens and its sister taxon *P. mulanjeensis* was 13.41%. These values fall within the range of uncorrected COI sequence divergence values reported between other southern African freshwater crab species (Daniels & Bayliss, 2012).

DISCUSSION

This study provides a description and phylogenetic placement of a novel endemic freshwater crab species from the Eastern Highlands of Zimbabwe. Although its distribution range overlaps that of *P. unispinus*, the species is both genetically and morphologically divergent from all three species currently known to occur in Zimbabwe (Fig. 2). This is the first discovery of an endemic species in Zimbabwe and brings the number of known freshwater crab species in Zimbabwe to four. The other three freshwater crab species, *P. bayonianus*, *P. obesus* and *P. unispinus*, are not endemic to Zimbabwe, but are widely distributed in southern Africa. This study serves as further evidence that there is still a great deal of hidden diversity in the Afrotropical region, especially in unsampled mountainous areas (Daniels & Bayliss, 2012) such as Malawi, Mozambique and Zimbabwe. With regards to

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freshwater crab diversity on the Eastern Highlands, there remains a large proportion of this mountain range, with a vast number of hydrological networks, which must still be sampled from both the Zimbabwean and Mozambican side, before a firm conclusion on the diversity and endemicity of freshwater crab species can be determined.

Currently, the biogeography and phylogenetic affinities of sub-Saharan African freshwater crabs is relatively unknown, particularly for the genus *Potamonautes*. However, owing to their generally limited dispersal abilities, most primary freshwater taxa are endemic to certain ecoregions (Cumberlidge, 1999; Cumberlidge & Sternberg, 2002; Cumberlidge & Wranik, 2002; Cumberlidge *et al.*, 2009; Daniels, 2011). Understanding the historical biogeography of freshwater taxa can reveal possible historical connectivity between these habitats, especially in large river systems. A study is currently under way to investigate phylogenetic relationships among all sub-Saharan African freshwater crabs, with particular emphasis on evolutionary affinities in *Potamonautes*.

Although two of the sampled localities were outside Nyanga National Park, it is not surprising that *Potamonautes mutareensis* sp. nov. was discovered in a protected area, where there has been minimal land transformation (as a result of deforestation and agriculture). The findings of this study represent a novel species of freshwater crab that is new to science. This is an important discovery as it will aid in resolving the inadequately resolved phylogeny of sub-Saharan African freshwater crabs. Moreover, the use of mtDNA-based phylogenies is proving to be of considerable value in both detecting novel evolutionary lineages and validating existing taxonomic units. Renewed systematic focus needs to be directed towards mountainous regions in the subcontinental region as these areas clearly harbour high levels of endemism. A dichotomous key to the freshwater crabs of Zimbabwe is provided.

SYSTEMATICS

SUBORDER BRACHYURA LINNAEUS, 1758

SUPERFAMILY POTAMOIDEA ORTMANN, 1896

FAMILY POTAMONAUTIDAE BOTT, 1970

SUBFAMILY POTAMONAUTINAE BOTT, 1970

GENUS *POTAMONAUTES* MACLEAY, 1838

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POTAMONAUTES MUTAREENSIS SP. NOV.

MATERIAL EXAMINED

Holotype: Mutare (eastern Zimbabwe), Odzani River (upper), forested valley in Nyanga mountains (Eastern Highlands mountain range; 1347 m a.s.l.), 18°49'52.6''S, 32°41'14.8''E, 1 ♂, SAM A45932 collected on 15.12.2011 by E. E Phiri, T. Dalu, E. Tambara and J. Mugabe. Endemic to the Eastern Highlands mountain range in Zimbabwe, near the Zimbabwe–Mozambique border and approximately 40 km south-east of Nyanga National Park (NNP – formerly Rhodes Inyanga National Park). Although not sympatric, its distribution overlaps with that of *P. unispinus*.

Paratype: Nyadiri River (18°22'7.5''S, 32°38'25.8''E, 1750 m a.s.l.), forested valley in Nyanga mountains, NNP (Eastern Highlands mountain range), SAM A45933, 2 ♂ collected on 14.12.2011 by E. E Phiri, T. Dalu, E. Tambara and J. Mugabe.

Additional material examined: Odzi River (18°22'7.5''S, 32°38'25.8''E, 1 428 m a.s.l., in NNP), SAM A45934, small Nyatanda River (18°21'9.9''S, 32°31'53.6''E, 1664 m a.s.l., in NNP), SAM A45935, and Chidiya River (18°18'51.3''S, 32°30'54.9''E, 1458 m a.s.l., *c.* 6.5 north-west of NNP), SAM A45936, 2 ♀ (one gravid) and 1 ♂ collected on 14.12.2011 by E. E. Phiri, T. Dalu, E. Tambara and J. Mugabe.

Diagnosis: Carapace vaulted and narrow, posteriorly. Epibranchial teeth absent and the anterolateral margin of carapace smooth. Postfrontal crest complete. Carapace chocolate brown. Dactylus of right cheliped highly arched with a row of small teeth.

Description: Potamonautes mutareensis sp. nov. has a high carapace depth (CH/CL = 0.55) and small posterior margin width (CWP/CL = 0.51). Measurements of the holotype (Fig. 3A, B, C) are presented in Table 1. Living specimen carapace, chelipeds, pereopods bright orange. Gonopod 1 has a terminal article *c.* 0.25 – 0.33 mm in length compared with the subterminal segment, while gonopod 2 has a long flagellum *c.* 0.5 – 0.75 mm times as long as the subterminal segment. Postfrontal crest complete. Carapace anterolateral margin smooth, lacks granulation or dentition (Fig. 3A). Exorbital tooth pointed and low. Suborbital margin slightly granulated. Deep urograstic groove. Pereopods short, narrow; pereopod 3 longest. Triangular abdomen widest at segment 3, narrowest at telson (Fig. 3B). Terminal segment of gonopod 1 (posterior view) points away from midline, terminal point curves upwards (Fig. 4A). Subterminal segment of gonopod 1 *c.* 2.5 mm at widest point. Subterminal of gonopod 1 basal margins have numerous setae (Fig. 4A, B). Terminal segment of gonopod 2 (posterior view) slanted away from midline at *c.* 10° angle (Fig. 4C). Subterminal segment

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of gonopod 2 approximately two-thirds of gonopod 1. Heterochelous chelipeds in males; right cheliped markedly larger. Right cheliped dactylus thin and arched (Fig. 5). Chelipeds equal sized in females. Cheliped teeth have few blunt grooves or serrations. Mandibular palp two-segmented, translucent with setae on periphery. Third maxilliped exopod has long flagellum (Fig. 6).

Distribution: The species appears to be restricted to first and second order streams in Mutare and on Mount Nyangani in the northern Eastern Highlands of Zimbabwe. Three out of the five localities fall within the Nyanga National Park on the slopes of Mount Nyangani. Although not sympatric, outside of the Nyanga National Park, the species' distribution overlaps with that of *P. unispinus*. In particular, there are at least five tributaries between Odzi River and Odzani River (a distance of *c*. 40 km) where *P. unispinus* occurs.

Remarks: While phylogenetic reconstruction results revealed that *P. mutareensis* sp. nov. is a sister taxon to *P. mulanjeensis* Daniels & Bayliss 2012, these two species bear no morphological resemblance. *Potamonautes mutareensis* sp. nov. has a vaulted carapace, while the carapace of *P. mulanjeensis* is flat. Compared with other east African freshwater crab species, both *P. mutareensis* sp. nov. and *P. mulanjeensis* are small-bodied species (CL = 25.26 mm and 25.38 mm, respectively). However, these species are geographically discrete, with *P. mulanjeensis* occurring in Malawi. The mandibular pulp of the two species is highly dissimilar. *Potamonautes mutareensis* sp. nov. superficially resembles other medium-bodied mountain-dwelling freshwater crab species occurring in east Africa, and likely represent convergent evolutionary characteristics. The phylogenetic results clearly demonstrate that the east African species are distinct from the southern African species (Fig. 2). Moreover, there are clear morphological distinctions between *P. mutareensis* sp. nov. and the three other freshwater crab species currently known to occur in Zimbabwe. The CWW of *P. mutareensis* sp. nov. is noticeably smaller (CWW = 34.81 mm) compared with *P. unispinus* (CWW = 49.83 mm) (Stewart & Cook 1998), *P. obesus* (CWW = 49.1 mm) (Reed & Cumberlidge 2004) and *P. bayonianus* (CW = 59.85 mm) (Cumberlidge 1997). Ecologically, the four species that occur in Zimbabwe are also distinct. *Potamonautes mutareensis* sp. nov. occurs in mountain streams, *P. unispinus* and *P. bayonianus* both occur in large drainage systems, primarily rivers, while *P. obesus* is generally found in ephemeral freshwater bodies such as pans, where it burrows into the sand bank. The cheliped of *P. obesus* is specifically adapted for burrowing, with the palm being flat. In addition, in all three of the described species from Zimbabwe, a tooth is present on the anterolateral margin of the carapace and varies in size

between the three species, while a tooth is absent on the anterolateral carapace margin in *P. mutareensis* sp. nov.

Etymology: The species is named after Mutare (Zimbabwe), the nearest town to where the holotype was collected.

Conservation: The species appears to be endemic to this mountain range, but may potentially have a wider distribution range in Zimbabwe and neighbouring Mozambique. The highlands are generally well conserved and several nature conservation areas are present, suggesting the species is currently well protected.

KEY TO THE FRESHWATER CRABS OF ZIMBABWE

1. Tooth absent from the epibranchial corner of the anterolateral margin of the carapace, carapace smooth……………………………………………………………*P. mutareensis*

A single tooth present on the epibranchial corner of the carapace, anterolateral margin sometimes dentate……………………………………………………………2

2. Postfrontal crest incomplete posterior to exorbital teeth, sloping distinctly backwards to meet epibranchial tooth, small but distinct tooth with epibranchial sinus anterior to tooth……………………………………………………………*P. obesus*

Postfrontal crest complete, sloping very slightly backwards to meet epibranchial tooth, no epibranchial sinus anterior to tooth……………………………………………3

3. Exorbital tooth as well as tooth on the anterolateral margin of the carapace spine-like……………………………………………………………*P. unispinus*

Exorbital tooth sharp, and the tooth on the anterolateral margin of the carapace broad and flat……………………………………………………………*P. bayonianus*

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Table 1 *Potamonautes mutareensis* sp. nov. Morphological character measurements (in mm) of the holotype and additional material (males and females separate) examined.

Character	Abbreviation	Holotype	Males	Females
Carapace length	CL	25.26	11.07 - 25.26	17.78 - 26.05
Carapace width at widest point	CWW	34.81	15.00 - 34.81	24.16 - 34.92
Carapace posterior margin	CWP	12.97	6.19 - 12.97	9.08 - 13.38
Frontal width	FW	3.61	1.32 - 3.61	2.41 - 3.96
Distance between postfrontal crest and anterior margin	PFCD	24.04	13.54 - 24.04	16.73 - 24.05
Carapace height	CH	13.84	5.46 - 13.84	9.50 - 14.29
Major cheliped propodus length	MCPL	32.11	8.79 - 32.11	13.97 - 23.49
Major cheliped propodus height	MCPH	13.33	2.89 - 13.33	5.87 - 9.20
Pereopod 2, merus length	P2ML	13.81	6.22 - 13.81	9.25 - 13.63
Pereopod 2, merus width	P2MW	5.35	1.98 - 5.35	3.71 -5.32
Pereopod 5, merus length	P5ML	12.47	6.67 - 12.47	9.54 - 12.41
Pereopod 5, merus width	P5MW	4.31	2.40 - 4.31	3.48 - 4.53

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Table 2 Substitution models and information regarding each model (obtained from jModelTest v. 2.1.3).

Gene fragment	No. of base pairs	Model	Base pair frequencies	Rate matrix	G distribution shape parameter	Proportion of invariable sites (I)
12S rRNA	318	TPM1uf + G	A = 39.35% C = 6.27%, G = 15.39%, T = 38.99%,	R (a) [AC] = 0.50 R (b) [AG] = 5.41 R (c) [AT] = 0.50 R (d) [CG] = 1.00 R (e) [CT] = 5.41 R (f) [GT] = 1.00	0.35	-
16S rRNA	435	TrN + I + G	A = 40.84% C = 5.71% G = 15.32% T = 38.13%	R(a) [AC] = 1.00 R(b) [AG] = 3.68 R(c) [AT] = 1.00 R(d) [CG] = 1.00 R(e) [CT] = 2.70 R(f) [GT] = 1.00	0.33	0.093
COI	569	TIM3 + G	A = 33.01% C = 15.68% G = 17.81% T = 33.51%	R(a) [AC] = 0.40 R(b) [AG] = 8.96 R(c) [AT] = 1.00 R(d) [CG] = 0.39 R(e) [CT] = 3.48 R(f) [GT] = 1.00	0.20	-

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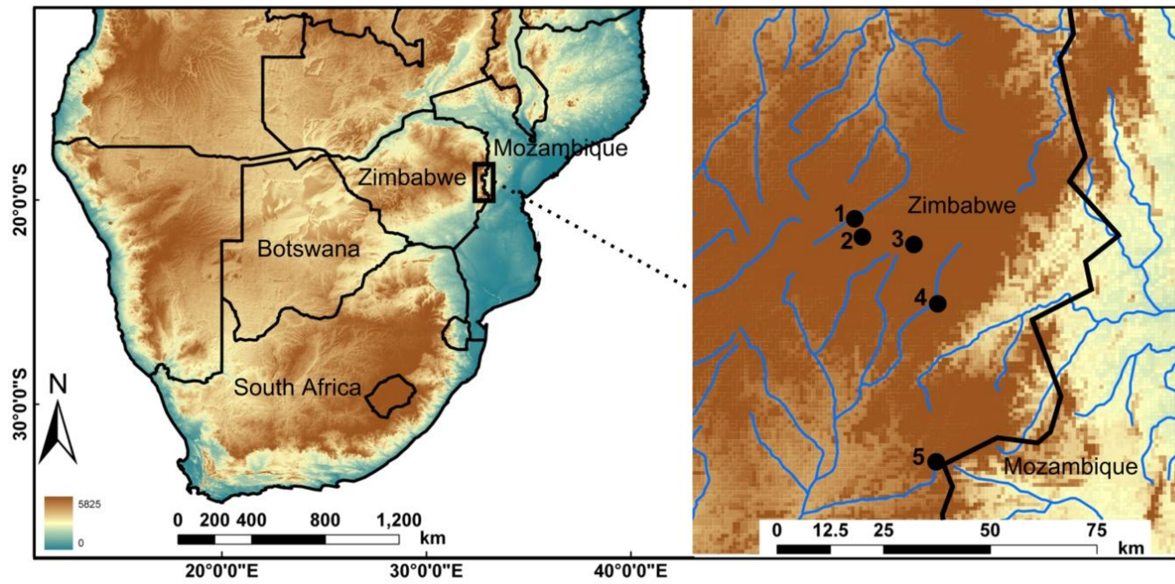

Figure 1 Tributaries of the Save drainage system on the Zimbabwean highlands of southern Africa where the freshwater crab specimens were collected: (1) Chidiya River; (2) Nyatanda River (small); (3) Nyadiri River; (4) Odzi River; (5) Odzani River (upper).

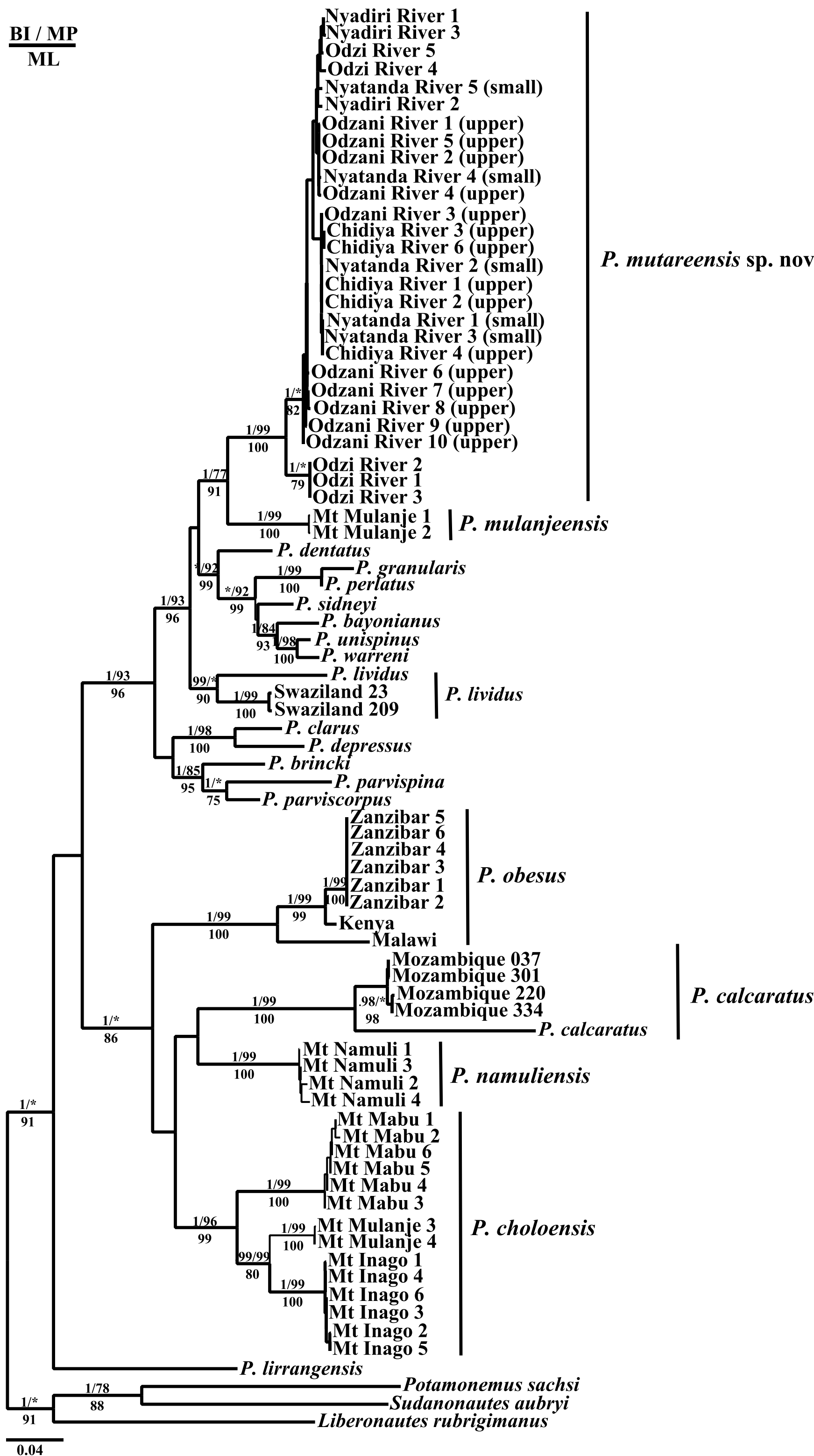

Figure 2 A maximum likelihood (ML) representation of the phylogenetic placement of the undescribed Zimbabwean freshwater crab specimens constructed from the combined 12S rRNA, 16S rRNA and COI. Node support is indicated by bootstrap values ≥75% for MP and ML, posterior probability values 0.95 for BI. An asterisk is indicative of nodes that are not statistically supported.

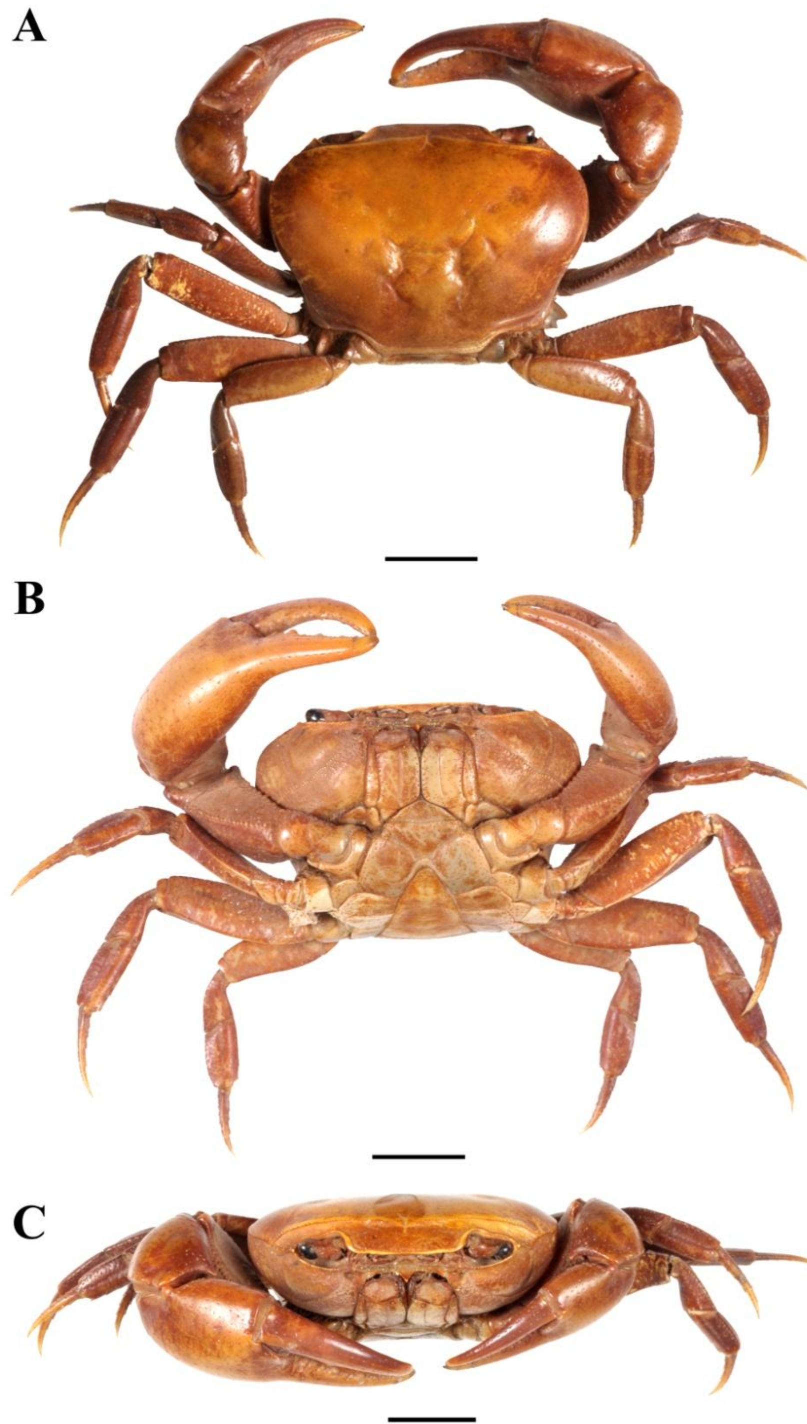

Figure 3 *Potamonautes mutareensis* sp. nov. male holotype (SAM A45932) (carapace length = 25.26 mm) from a forested valley in the Nyanga mountains (1500 m a.s.l., Mutare, eastern Zimbabwe), Eastern Highlands mountain range: (A) dorsal aspect; (B) ventral aspect; (C) cephalothorax, frontal aspect. Scale bars = 10 mm.

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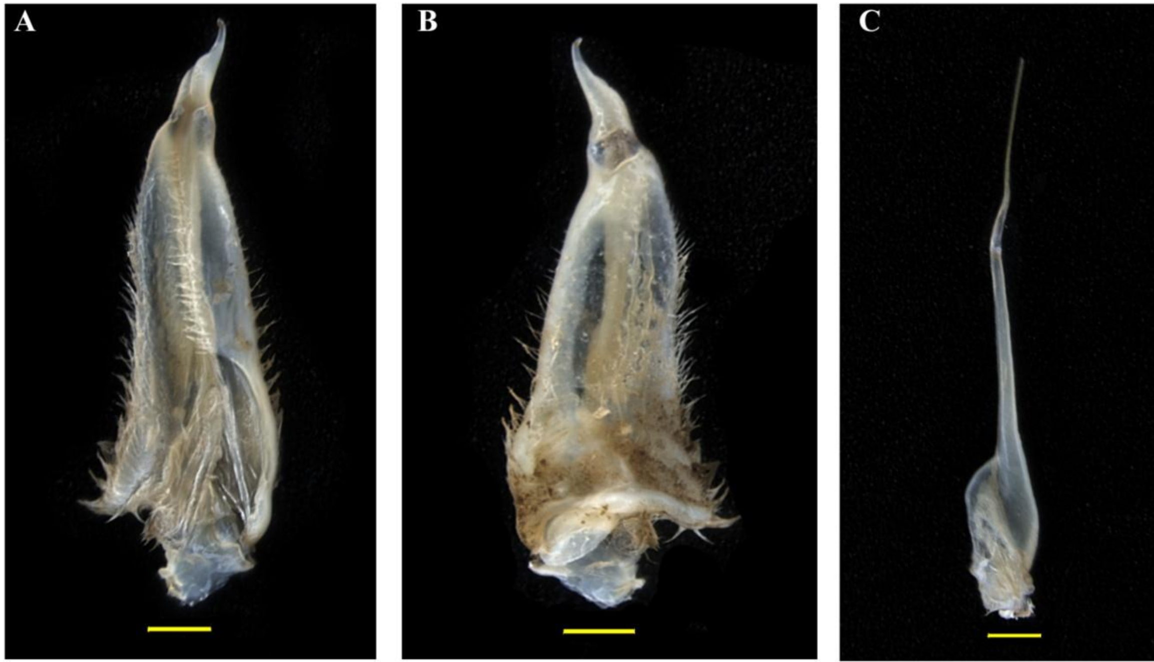

Figure 4 *Potamonautes mutareensis* sp. nov. male holotype (SAM A45932): (A) left gonopod 1, anterior view; (B) left gonopod 1, posterior view; (C) left gonopod 2, posterior view. Scale bars = 1.0 mm.

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Figure 5 Right and left chelae of *Potamonautes mutareensis* sp. nov. male holotype (SAM A45932). Scale bar = 10 mm.

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Figure 6 Left third maxilliped of *Potamonautes mutareensis*, sp. nov. male holotype (SAM A45932). Scale bar = 1.0 mm.

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CHAPTER 5

RENEWED SAMPLING OF INLAND AQUATIC HABITATS IN SOUTHERN AFRICA YIELDS TWO NOVEL FRESHWATER CRAB SPECIES (DECAPODA: POTAMONAUTIDAE: *POTAMONAUTES*)*

ABSTRACT

A recent survey of the freshwater streams of the Mecula and Yao Mountains in the Niassa Province of Mozambique resulted in the discovery of a new freshwater crab species. This species is genetically and morphologically distinct from described species from Mozambique or its neighbouring countries, and is described as *Potamonautes bellarussus* sp. nov. In addition, a new semi-terrestrial burrowing freshwater crab *Potamonautes flavusjo* sp. nov. from the Highveld of the Mpumalanga Province in South Africa is described based on unique genetic and morphological characters. The phylogenetic affinities of the two new species in relation to the described eastern and southern African *Potamonautes* species is determined and the biogeographic implications discussed.

INTRODUCTION

Afrotropical freshwater crabs as a whole are species-poor compared to other biogeographic regions such as the Neotropical and Oriental areas (Yeo *et al*., 2008; Cumberlidge *et al*., 2009). Freshwater crab diversity in rivers, first and second order streams and lakes in the Afrotropics is well documented, but aquatic habitats in remote mountainous regions have been neglected, hindering a more accurate reflection of species diversity and endemism. Recent sampling of freshwater habitats in the Afrotropical region has yielded several new species of potamonautid freshwater crab (for example, Cumberlidge & Dobson, 2008; Cumberlidge & Tavares, 2006; Cumberlidge & Clark, 2010; Duris & Koch, 2011; Cumberlidge & Meyer, 2011; Meyer & Cumberlidge, 2011; Cumberlidge & Clark, 2012; Daniels & Bayliss, 2012; Phiri & Daniels, 2013). In southern Africa, Daniels & Bayliss (2012) discovered two new species of freshwater crabs from mountainous regions in Malawi and Mozambique. A recent survey of streams on the eastern Zimbabwean highlands resulted in the discovery of a new species of *Potamonautes* which is the first endemic species reported

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from Zimbabwe (Phiri & Daniels, 2013). Cumberlidge & Clark (2012) described the first cave-living potamonautid freshwater species from the Ethiopian highlands. Collectively, these results reiterate the renewed call for poorly sampled high altitude freshwater systems in the Afrotropics to be a focal point of future systematic surveys.

The present work reports on the presence of a new freshwater crab species from the Mecula and Yao mountains, in the Niassa Province in northern Mozambique that were sent to the senior author for identification. These specimens were collected by J. Bayliss whilst undertaking an expedition to investigate unexplored mountainous regions (>1500m) in this region of Africa and to determine the biogeographical linkages of species. Six freshwater crab species are known from Mozambique; *Potamonautes bayonianus* Brito-Capello, 1873, *P. calcaratus* Gordon, 1929, *P. choloensis* Chace, 1953, *P. namuliensis* Daniels & Bayliss, 2012, *P. obesus* H. Milne Edwards, 1868 and *P. sidneyi* Rathbun, 1904. Only the recently described species *P. namuliensis* from the Namuli Mountains is endemic to Mozambique. The presence of a single endemic freshwater crab species reflects the poorly sampled nature of inland aquatic systems in Mozambique, particularly since several isolated mountains are present in the rugged interior of the country. Morphological comparisons of the Mecula and Yao Mountain specimens with the six described Mozambican freshwater crab species, and with species from Malawi and Tanzania suggest that the Mecula and Yao specimens represent a undescribed species.

In contrast to the Mozambican freshwater crab fauna, the freshwater crab systematics in neighbouring South African is well studied. The South African inland decapod fauna has been subjected to rigorous systematic scrutiny (using allozymes, mtDNA sequence data, morphology and morphometrics) resulting in the description of seven novel species (Stewart *et al*., 1995; Stewart & Cook, 1998; Stewart, 1997; Daniels *et al.,* 1998; Gouws *et al*., 2000, 2001; Daniels *et al*., 2001). South Africa's freshwater crab fauna currently comprise 13 described species; *P. brincki* Bott, 1960, *P. calcaratus* Gordon, 1929, *P. clarus* Gouws, Stewart & Coke, 2000, *P. dentatus* Stewart & Cook, 1995, *P. depressus* Krauss, 1843, *P. granularis* Daniels, Stewart & Gibbons, 1998, *P. lividus* Gouws, Stewart & Reavell, 2001, *P. parvispina* Stewart, 1997, *P. parvicorpus* Daniels, Stewart & Burmeister, 2001, *P. perlatus* H. Milne-Edwards, 1837, *P. sidneyi* Rathbun, 1904, *P. unispinus* Stewart & Cook, 1998, and *P. warreni* Calman, 1918. More recently several cryptic lineages have been identified and are being described (E.E. Phiri pers. obs.). The primary focus of these earlier systematic endeavours was to document the diversity of freshwater crabs in rivers and mountain streams, however, non-riverine habitat, such as vlei (wetland) areas have been largely neglected.

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Dr Johan Engelbrecht formerly of the Mpumalanga Parks sent an ethanol preserved female specimen of an unidentified freshwater crab species from Verloren Vallei Nature Reserve, to the first author for identification during December 2000. Since the gonopods of male specimens are required for species identification, the female specimen could not be conclusively identified. However, the female specimen was recently subjected to mtDNA sequencing, and the resultant sequences were compared to all the described southern African freshwater crab species (Daniels *et al*., 2002). Preliminary analyses of the mtDNA sequence study unambiguously demonstrated that the Verloren Vallei Nature Reserve freshwater crab specimen represent a new species. More recently, Jerry Theron and Gerhard Diedericks sent a series of photographic images of a freshwater crab specimen similar in appearance to the female specimen from Verloren Vallei Nature Reserve that was collected in the Chrissiesmeer district. Both of the latter localities occur in the Highveld of the Mpumalanga Province in South Africa. The Highveld is an inland plateau with an altitude above 1500 m but below 2100 m. The presence of the undescribed Highveld freshwater crab species resulted in the senior author undertaking a sampling trip to the region to collect male specimens of the species.

The objectives of the present study are twofold. Firstly, to diagnose and described the novel Mozambican and South African freshwater crab species in comparison to the known species from east and southern Africa. Secondly, to determine the phylogenetic placement of the two novel species in relation to the described species from east and southern Africa with the use of three partial mtDNA loci.

MATERIALS AND METHODS

SAMPLE COLLECTION

Freshwater crabs were collected from the Mecula (n = 4) and Yao (n = 2) Mountains in the Niassa Province of Mozambique (Fig. 1). These crabs were hand collected from large bolder strewn mountain streams in forested areas. The latter undescribed freshwater crab species from Mozambique closely resembles *P. suprasulcatus* (Hilgendorf, 1898). Hence two specimens of *P. suprasulcatus* from Malagarasi in Tanzania, were included in this present study. The latter specimens were obtained from the British Natural History Museum, London, United Kingdom and send to the first author by Neil Cumberlidge for a larger phylogenetic study on the Afrotropical freshwater crabs.

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The undescribed Highveld species was collected from three sample sites at Verloren Vallei Nature Reserve (A, B and C), Elandshoek, as well as the two farms Iona, and Miss Chrissie's Country House Guest farm, Chrissiesmeer in the Lake Chrissiesmeer district in the Mpumalanga province of South Africa. At the two Verloren Vallei Nature Reserve (B and C) sample sites as well as at the two farms in the Lake Chrissiesmeer district, the undescribed Highveld species was sympatric with *P. sidneyi*. A total of 18 *P. sidneyi* specimens and 28 specimens of the undescribed Highveld freshwater crab species were collected (Fig. 1). The undescribed Highveld freshwater crab species is a semi-terrestrial burrowing freshwater crab. Burrows were generally present under decaying Poaceae (grass) and Restionaceae (restio's / reeds) in peat soil in close proximity to small streams. Active burrows were characterised by the deposition of moist soil at the burrow entrance and these burrows were frequently sealed with a peat plug. Freshwater crabs were dug from their burrows using a garden spade. Burrow depth depended on the depth of the water table and was variable. A handheld GPS was used to record the latitude and longitude where samples were collected. Crabs were killed by freezing overnight and leg tissue was preserved in absolute ethanol for DNA analyses.

DNA EXTRACTION, PCR, AND SEQUENCING

Muscle tissue extracted from walking legs was subjected to DNA extraction using a Qiagen DNEasy kit, following the manufacturer's protocol. Extracted DNA was stored in a refrigerator until required for PCR. Generally, a 1µL DNA in 19µL water dilution was performed prior to use. Three partial gene fragments were selected for the present study, these included the cytochrome oxidase subunit one (COI), 12S rRNA and 16S rRNA. These three loci were selected because each has a different mutation rate and had been successfully used for reconstructing evolutionary relationships among freshwater crabs (Daniels *et al.*, 2002a, 2006a, b; Daniels & Bayliss, 2012). Primer pairs used are outlined in Daniels *et al.* (2002a, 2006a). Standard PCR conditions were followed for amplification and DNA sequencing protocols were followed (Daniels *et al.*, 2006a).

PHYLOGENETIC ANALYSES

Sequence Navigator (Applied Biosystems) was used to compute a consensus sequence from forward and reverse strands. No insertions or deletions were evident for the protein coding COI locus and sequences for this locus were aligned manually. The 12S rRNA and 16S rRNA loci were aligned using Clustal X (Thompson *et al.*, 1997). Since all three partial

fragments occur on the mitochondria and are linked, I combined the mtDNA sequence data into a single data matrix and conducted all analyses on the combined data set. Maximum Parsimony (MP) and Bayesian approaches were used to estimate evolutionary relationships. MP analyses were executed in PAUP*4 version beta 10 (Swofford, 2002). For the MP analyses, trees were generated using the heuristic search option with TBR branch swapping using 100 random taxon additions, with gaps treated as fifth characters in the 12S rRNA and 16S rRNA. Phylogenetic confidence in the nodes recovered from MP was estimated by bootstrapping (Felsenstein, 1985), analyzing 1000 pseudoreplicates of data sets. Bootstrap values for nodes of < 75% were regarded as poorly resolved. Uncorrected sequence ("p") distances were calculated in PAUP*4 version beta 10 (Swofford, 2002). ModelTest version 3.06 (Posada & Crandall, 1998) was used to obtain the best-fit substitution model for each gene locus. These substitution models were used in the partitioned Bayesian analyses. The best-fit maximum likelihood score was chosen using the Akaike information criteria (AIC) (Akaike, 1973), since this reduces the number of parameters that contribute little to describing the data by penalizing more complex models (Posada & Buckley, 2004; Nylander *et al*., 2004). Bayesian inferences were used to investigate optimal tree space using the program MrBayes v. 3.0b4 (Ronquist & Huelsenbeck, 2003). For each analysis, four Markov chains were run, with each chain starting from a random tree and run for 5×10^6 generations, sampling each chain every 1×10^3 trees. This process was repeated four times to ensure that trees converged on the same topology. A 50% majority rule consensus tree was generated from the trees retained (after the burn-in trees were discarded – using likelihood plots) with posterior probabilities (p*P*) for each node estimated by the percentage of time the node was recovered. Posterior probability (p*P*) values <1.00 was regarded as poorly resolved. The combined data were analysed using the mixed substitution models for each locus in the Bayesian analyses.

Daniels *et al*. (2006a) demonstrated that *Liberonautes* (Bott, 1955) is sister to *Potamonautes*. Hence four *Liberonautes* (Bott, 1955) species were used as outgroups, *L. latydactylis* (De Man, 1903), *L. lugbe* (Cumberlidge, 1999), *L. nimba* (Cumberlidge, 1999) and *L. rubrigimanus* (Cumberlidge & Sachs, 1989). The GENBANK accession numbers of the outgroup species and the described South African freshwater crab species are provided in Daniels & Bayliss (2012). GENBANK sequences for the newly described species *P. mutareensis* (Phiri & Daniels, 2013) from Zimbabwe are provided by Phiri & Daniels (2013).

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MORPHOLOGY

Samples were divided into males and females and the following measurements were taken with digital vernier callipers. The carapace length (CL); the carapace widest at width point (CWW); the width of the posterior margin of the carapace (CWP); the distance between the postfrontal crest and the anterior margins of the carapace (PFCD); the frontal width measured between the medial margins of the orbits (FW); and the carapace height (CH), pereiopod measurements were also take, length and width of merus of pereiopod 2 and 5, the length of the dactylus of the major cheliped. All measurements are given in mm. Samples have been deposited in the South African Museum of Natural History, Iziko Museums of Cape Town, South Africa (SAM). Freshwater crabs were photographed with the use of a Nikon A 300 digital camera. In addition, the structure of gonopods 1 and 2 were photographed with a Leica MZ 75 digital camera, attached to a Leica EC 3 X stereo-microscope.

RESULTS

The combined mtDNA data yielded a total of 1278 base pairs (bp) that comprised 316 bp, 362 bp and 600 bp for the 12S rRNA, 16S rRNA and COI gene regions respectively. A total of 138 mtDNA sequences were generated during the present study. Sequences of the three partial mtDNA gene fragments were deposited in GENBANK, 12S rRNA (XXX-XXX), 16S rRNA (XXX-XXX) and COI (XXX-XXX). The MP analyses retrieved 116 trees, with CI = 0.40, RI = 0.78 with a tree length of 2149 steps from 426 parsimony informative characters. The three substitution models (used for the BI analyses) retrieved using ModelTest are listed in Table 1. The tree topology for the two analytical methods (MP and BI) retrieved near identical tree topologies, hence only the BI topology is shown (Fig. 2). *Potamonautes* was retrieved as monophyletic (MP = 84%, p*P* = 1.00) based on the present taxonomic sampling. A basal, statistically well-supported clade (MP = 84%, p*P* = 1.00) comprised *P. obesus*, *P. namuleensis*, *P. calcaratus* and *P. choloensis* was retrieved. All four species occur in Mozambique, while the specimens from the Mecula and Yao Mountains in the Niassa Province of Mozambique were retrieved as sister and formed a distinct clade (MP = 90%, p*P* = 1.00). The latter clade was distantly related to the four aforementioned freshwater crab species from Mozambique that comprised the basal clade. Despite the morphological similarity of the freshwater crab specimens from the Mecula and Yao Mountains to *P. suprasulcatus,* these two species were not closely related. *Potamonautes*

suprasulcatus was retrieved as sister to *P. lirrangensis* with strong statistical support (MP = 96%, p*P* = 1.00), while the latter clade was sister (ML = 90%, p*P* = 1.00) to *P. platynotus* and *P. raybouldi* (MP = 87%, p*P* = 1.00) forming a distinct east African freshwater crab clade that is basal to the southern African freshwater crab taxa.

The specimens from the, Mpumalanga Highveld in South Africa fell into two distinct clades. The 18 *P. sidneyi* specimens from the two sample sites (Verloren Vallei Nature Reserve B and C) together with specimens from the farm Iona and the one specimen from the the Miss Chrissie's Country House, Chrissiemeer formed a clade (clade B) (MP = 83%, p*P* = 1.00). The latter clade was in turn sister to *P. sidneyi* (ML <75%, p*P* = 1.00). The entire *P. sidneyi* clade (clade B) was sister to all the large bodied southern African riverine freshwater crab species (MP <75%, p*P* = 1.00). The undescribed specimens from the Highveld collected at Verloren Vallei Nature Reserve A, B and C the farm Iona and the Miss Chrissie's Country House Guest farm, Chrissiesmeer formed a distinct clade (clade A) (MP = 100%, p*P* = 1.00). In the Bayesian analyses the undescribed Highveld freshwater crab species in (clade A) was retrieved as sister to *P. mulanjeensis*, with poor statistical support (MP <75%, p*P* = 1.00). The latter clade was in turn sister to *P. mutareensis* and *P. dentatus* (MP <75%, p*P* = 1.00). Uncorrected “p” distances between *P. sidneyi* (clade B) and the sympatric novel Highveld lineage (clade A) exhibited a 13.03% divergence for the partial COI mtDNA locus. These results corroborate the reproductive isolation of the two maternal gene pools where *P. sidneyi* specimens occur in sympatry with the undescribed Highveld freshwater crab species.

DISCUSSION

The results indicate that even well-sampled countries such as South Africa can still yield novel freshwater crab species. Other Afrotropical countries that have not recently been subjected to surveys of their inland freshwater regions are similarly likely to harbour a wealth of undocumented freshwater crab diversity. For example, in Mozambique where in the last two years two novel endemic species (*P. bellarussus* sp. nov and *P. namuliensis*) have been recorded from remote areas of Mozambique, while the distribution of another species (*P. choloensis*) has been extended, and a new species from Gorongosa National Park in Mozambique is currently being described (Cumberlidge pers. comm.). Similar hidden diversity patterns are likely to be true for Zimbabwe following the recent discovery of that country’s first endemic freshwater crab species, *P. mutareensis*. A systematic focus on inland freshwater systems such as ephemeral pans, caves, remote inaccessible mountain streams and

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waterfalls in poorly sampled areas could potentially yield several novel freshwater crab species as well as other freshwater taxa, such as freshwater fishes, dragonflies, and molluscs. The East African Rift Valley has a tremendous diversity of freshwater crab species, particularly in Kenya and Tanzania, and has historically been well-sampled (Reed & Cumberlidge, 2006; Cumberlidge & Dobson, 2008; Cumberlidge & Clark, 2010; Cumberlidge & Meyer, 2011). However, the southern regions of the mountain ranges in Mozambique and most of the adjoining Zimbabwe Highlands are among the most poorly sampled areas on the continent and are likely to harbour equally high levels of endemism.

The distinctiveness together with the marked genetic divergence of COI among the sympatric *P. sidneyi* and *P. flavusjo* sp. nov at four of the sample sites on the Highveld corroborates the distinctiveness of species. Where *P. sidneyi* with *P. flavusjo* sp. nov are sympatric, no haplotypes were shared between the two species, providing evidence of reproductive maternal isolation between the two species, when invoking the biological species concept. Furthermore, the two species exhibit fixed morphological differences in gonopods 1 and 2 and carapace morphology, providing additional support for the phylogenetic species concept. In addition, *P. flavusjo* sp. nov and *P. sidneyi* are ecologically distinct. *Potamonautes flavusjo* sp. nov., is an extensive burrowing species that occurs exclusively in peat soils where restio's (reeds) and grass pools are present, while *P. sidenyi* is widely distributed in rivers and streams from the Eastern Cape, KwaZulu-Natal, Mpumalanga, and Limpopo provinces of South Africa and only digs shallow burrows. For a burrowing species, *P. flavusjo* sp. nov., exhibits low levels of genetic variation over the sampled areas in the Highveld. The shallow genetic differentiation is rather surprising since the two burrowing Afrotropical freshwater crab species that have been subjected to population level genetic analyses (*P. calcaratus* and *Seychellum alluaudi* (A. Milne-Edwards and Bouvier, 1893)) exhibit deep genetic differentiation at small spatial scales (Daniels *et al*., 2002b; Daniels, 2011). These results suggest the potential presence of unsampled subpopulations of *P. flavusjo* sp. nov., within the sampled area on the Highveld, or alternatively the results are indicative of a highly amphibious capability for this semi-terrestrial species in the absence of major barriers to gene flow. I am unable to differentiate between these two hypotheses, however it is likely that during periods of heavy rain during the summer months, the species is capable of widespread overland dispersal considering its adaptation to a semi-terrestrial mode of life. Ecological validation of the latter observations is required.

Interestingly, *P. sidneyi* is a morphologically variable taxon, for example, the Verloren Vallei *P. sidneyi* specimens were chocolate brown in colour, with a slightly vaulted carapace (likely due to its burrowing mode of life), while the Chrissiesmeer district *P. sidneyi* specimens exhibited a near purple carapace margin and chelipeds and a flat carapace typical of river living animals. Despite the variation in morphology these specimens formed a single statistically well-supported clade (clade B), suggesting that single morphological characters alone should be treated with caution when inferring species boundaries.

Phylogenetically, *P. flavusjo* sp. nov., from the Highveld in Mpumalanga is not closely related to any of the described South African species, instead it is sister to *P. mutareensis* from Zimbabwe and *P. mulanjeensis* from Malawi. Biogeographically the north eastern parts of Southern Africa is well known to harbour species with a strong tropical affinity, and the close phylogenetic relationship of *P. flavusjo* sp. nov., with taxa outside of South Africa corroborates evidence for a northern tropical ancestry. The northeastern region of South Africa (including the KwaZulu-Natal, Limpopo and Mpumalanga provinces) represents the southern limits of the distribution of numerous tropical species. The results suggest that the current aquatic boundaries that were historically used to define the southern African fauna region do not correspond with freshwater ecoregions and requires revision.

SYSTEMATICS

SUBORDER **BRACHYURA** LINNAEUS, 1758

SUPERFAMILY **POTAMOIDEA** ORTMANN, 1896

FAMILY **POTAMONAUTIDAE** BOTT, 1970

SUBFAMILY **POTAMONAUTINAE** BOTT, 1970

GENUS ***POTAMONAUTES*** MACLEAY, 1838

POTAMONAUTES BELLARUSSUS SP. NOV.

(FIG. 3, FIGS. 4 A - C; FIGS. 5 A - C)

MATERIAL EXAMINED

Holotype: One ♂ specimen (SAM A48212), Yao Mountain, 1045 m altitude above sea level (a.s.l.), 12º27’276”S, 36º32’260”E, Niassa Province, northern Mozambique collected on 13. 05. 2012 by J. Bayliss. Specimens were collected from bolder strewn mountain streams in a mixed riverine / woodland habitat close to the summit.

Paratype: One ♂ specimen (SAM A48213), Yao Mountain same collection information as the holotype.

Additional material examined: One ♀ (SAM A48217), Yao Mountain 1045 m a.s.l., 12º27'276"S, 36º32'260"E, Niassa Province, northern Mozambique, collected on 13 May 2012 by J. Bayliss, one ♀ (SAM A48218) Yao Mountain 1045 m a.s.l., 12º27'276"S, E, Niassa Province, northern Mozambique, collected on 13 May 2012 by J. Bayliss, three ♀ specimens from the Mecula Mountain, Niassa Province 1046 m a.s.l. (SAM A48214, 48215 and 48216), at 12º43'206"S, 37º38'267"E, northern Mozambique, collected on 09. 05. 2012 by J. Bayliss.

Diagnosis: Flat freshwater crab species, postfrontal crest deep and well defined, exorbital tooth low but prominent, anterolateral margins granulate (Fig. 3, Figs. 4 A, B and C). Carapace, periopods, chelipeds blood red when alive (colour faded faded in preserved specimens).

Description: See Table 2 for the measurements of the holotype. Postfrontal crest deep, distinct, crossing the entire carapace, groove at posterior part of carapace deep. Colour faded in preserved specimens. Carapace flat (CWW / CH = 3.07), narrow posteriorly (CWP / CL = 0.44). Exorbital epibranchial tooth prominent, anterolateral margin behind epibranchial tooth heavily granulated. Anterolateral margins heavily granulated, urogastic and cardiac regions deep, well-defined, subhepatic region of carapace sidewall faintly granulated. Sternites of sulcus s3/s4 well defined. Dactylus of major cheliped with series of well defined teeth, dactylus not arched, tips white. First carpal tooth on carpus of cheliped with large prominent and sharp spine, second carpal tooth smaller, third small carpal tooth behind the second tooth is present but reduced in size. Terminal article of gonopod 1 long, slim curving to the right and comprise nearly half of gonopod 1 and comprise nearly half of the length of gonopod 1 (Figs. 5 A and B). Gonopod 2 is thin, long and filamentous, the terminal article long and slender with a slight curve (Fig. 5 C).

Distribution: Endemic to Mecula and Yao Mountains in the Niassa Province of northern Mozambique.

Remarks: Potamonautes bellarussus sp. nov., resembles *P. suprasulcatus* in morphology. *Telphusa suprasulcata* var. *pseudoperlata* (Hilgendorf, 1898); *Telphusa mrogoroense* (Hilgendorf, 1898) are considered here to be junior subjective synonyms of *P. suprasulcatus.* However, phylogenetically these two species are distantly related. *Potamonautes bellarussus* sp. nov., formed a distinct clade while *P. suprasulcatus* was sister to sister to *P. lirrangensis*. In addition *P. suprasulcatus* has a carpal tooth on carpus of cheliped sharp spine; second carpal tooth smaller spine, with no other teeth behind it, while a tooth is present in *P. bellarussus* sp. nov. In addition *P. bellarussus* sp. nov., and *P.*

suprasulcatus is ecologically distinct, with the former living in mountain streams while the latter species live in major rivers (Reed & Cumberlidge, 2006).

The novel Mozambican freshwater crab species was also phylogenetically distantly related to the six described freshwater crab species from Mozambique (*P. bayonianus*, *P. choloensis*, *P. calcaratus*, *P. obesus*, *P. namuliensis*, and *P. sidneyi*). Notably, the four east African freshwater crab species present in Mozambique (*P. choloensis*, *P. calcaratus*, *P. obesus*, *P. namuliensis*) formed a distinct basal clade, the two remaining species (*P. bayonianus* and *P. sidneyi*), characterised by wide distribution ranges in southern Africa belonged to a clade comprising the large bodied freshwater crab species. Superficially *P. bellarussus* sp. nov., also resembles *P. unisulcatus* (Rathbun, 1933). The latter species is endemic to the Uluguru Mountains in northern Tanzania, gonopod 1 of *P. bellarussus* sp. nov., is different from *P. unisulcatus* (Reed & Cumberlidge, 2006). In addition, in *P. bellarussus* sp. nov., three carpal teeth are present while in *P. unisulcatus* only two teeth are present.

Etymology: The name *P. bellarussus* sp. nov., is an arbitrary combination of two aspects. 'Bellus' is Latin for beautiful, and is in honour of Dr Bella Davies for her dedication to freshwater ecology in the United Kingdom and Africa. While the Latin 'russus' refers to the blood red in colour of living specimens. The name is used as a noun in apposition.

POTAMONAUTES FLAVUSJO SP. NOV.

(FIG. 6; FIGS. 7 A - C; FIGS. 8 A - C)

Holotype: One ♂ specimen (SAM A48203), Verloren Vallei Nature Reserve (B), 2000 m asl, 25º 20' 19" a.s.l., 25º20'333"S, 30º07'546"E, Mpumalanga Province of South Africa, collected on 27. 06. 2013 by S. Daniels, F. Gordon and P. Makuwa.

Paratype: Six ♂ and one ♀ specimen, (SAM A48204) Verloren Vallei Nature Reserve (A), 2018 m a.s.l., 25º18'412"S, 30º08'428"E, Mpumalanga Province of South Africa collected 28. 06. 2013 by S. Daniels, F. Gordon, P. Makgwa and G. Diedericks.

Additional material examined: Six ♀ (SAM with the same locality and collection information as the holotype. One ♀ (SAM A48207), Verloren Vallei Nature Reserve (A), 2016 m a.s.l., 25º18'422"S, 30º08'427"E, Mpumalanga Province of South Africa collected on 27. 06. 2013 by S. Daniels, F. Gordon and P. Makuwa. One ♂ (SAM), Verloren Vallei Nature Reserve (C), 2065 m a.s.l., 25º17'305"S, 30º09'030"E, Mpumalanga Province of South Africa collected on 28. 06. 2013 by S. Daniels, F. Gordon, P. Makgwa and G.

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Diedericks. One ♀ (SAM A48209), Elandshoek, 1987 m a.s.l., 25º22'030"S, 30º06'764"E, Mpumalanga Province of South Africa collected on 28. 06. 2013 by S. Daniels, F. Gordon and G. Diedericks. Nine specimens (SAM A48205), Miss Chrissie's Country House Guest Farm, Chrissiesmeer, next to Lake Chrissiesmeer, 1665 m a.s.l., 26º17'553"S, 30º13'414"E, Mpumalanga Province of South Africa collected on 30. 06. 2013 by S. Daniels and F. Gordon. Five specimens (SAM A48210) Farm Iona, in the Lake Chrissiesmeer district, 1648 m a.s.l., 26º13'777"S, 30º10'667"E, Highveld of the Mpumalanga Province of South Africa, collected on 02. 07. 2013 by S. Daniels, F. Gordon, H. Marais and U. Franke.

Diagnosis: Carapace highly vaulted, post frontal crest margin, periopods and chelipeds bright yellow, fading to dull yellow upon preservation in absolute ethanol. Anterolateral margin of carapace smooth, lacking dentition, exorbital tooth low but well defined (Fig.7 A-C). Ventral carapace surface and ventral chelipeds light yellow.

Description: See Table 3 for measurements of the holotype. Live specimens of *P. flavusjo* sp. nov., have a distinct yellow post frontal crest band and dull mottled yellow marked cephalograstic and anterolateral margin region. Chelipeds and ventral surface of periopods light yellow, dorsal surface of the pereopods is light brown (Figs. 7 A, B, C). Colour fades upon preserved. Carapace distinctly vaulted (CWW / CH = 2.33) narrow posteriorly (CWP / CL = 0.51). The anterolateral margin smooth. Postfrontal crest deep well defined and curves forward medially. Exorbital tooth, small moderately sharp. Urogastric and cardiac grooves defined. Sternites 1 and 2 fused. First suture between 2 and 3 complete. Second sternal groove between sternites 3 and 4 complete. Third maxillipeds fill the entire buccal frame, except for respiratory opening. Mandibular palp two segments, terminal segment undivided, sense tuft of setae on posterior surface of flange. Chelipeds generally unequal, in the holotype the right cheliped is broken off. Dactyli armed with 18 small cutting teeth. Carpi of chelipeds possess one prominent tooth and two small teeth. Pereopods slender, pereopod 3 is the longest, 5 the shortest. Dorsal margins of pereopods fine sharp bristles, dactyli ending in sharp points, margins possessing spine-like bristles. Pleopods 1 (gonopod 1), terminal segment was short, 0.24 times length of subterminal segment, terminal segment curves away from midline when viewed posteriorly, widest at base ending in point. Subterminal segment tapers distally (Figs. 8 A, B). Pleopod 2 (gonopod 2) terminal filament like, 0.5 times the length of subterminal segment (Fig. 8 C).

Distribution: Endemic to the Highveld region in the Mpumalanga Province of South Africa where the species occurs exclusively in vlei (wetland) areas next to small streams

where they burrow into peat soils. Burrows are always found at the base of grass pools and reed beds.

Remarks: Although frequently collected sympatrically with *P. sidneyi*, the new species, *P. flavusjo* sp. nov is genetically and morphologically distinct. Phylogenetically, *P. flavusjo* sp. nov., is sister to *P. mulanjeensis* from Mount Mulanje in Malawi and *P. mutareensis* from the Nyanga mountain range in the Eastern Highlands of Zimbabwe based on mtDNA evidence. These three species can easily be distinguished morphologically. *Potamonautes mutareensis* shows a moderately arched right dactylus, a characteristic common in most small bodied freshwater crab living in mountainous regions, the latter feature is absent in *P. flavusjo* sp. nov, however the dactylus is not arched in any of the other specimens. In addition the two species are also distinct on the basis of carapace colour, with *P. flavusjo* sp. nov., possessing a bright yellow post frontal crest margin, and yellow margins of the carapace, while these features in *P. mutareensis* are all brown. *Potamonautes mulanjeensis* is similar sized species (CWW = 34.81) to *P. flavusjo* sp. nov. Neither of the two species sister to *P. flavusjo* sp. nov., have a burrowing lifestyle.

Superficially, *P. flavusjo* sp. nov., is morphologically similar to the two South African freshwater crab species that are semi-terrestrial, *P. calcaratus* and *P. lividus*. All three freshwater crab species are characterised by a vaulted carapace, an adaptation to a semi-terrestrial life style away from permanent flowing rivers and streams. In both *P. lividus* and *P. calcaratus,* the dactylus of the right cheliped is arched while in *P. calcaratus* the dactylus of the right chela is flattened an adaptation for burrowing. *Potamonautes calcaratus* has a small but distinct tooth on the anterolateral margin of the carapace, and a near flat right cheliped, and a slate black carapace. The carapace of *P. lividus* when alive is blue with a light blue shine, while the chelipeds and limbs are orange or red. No carapace dentition is present in either *P. lividus* or *P. flavusjo* sp. nov.

In contrast in *P. flavusjo* sp. nov., the major cheliped shows no special adaptation for a burrowing mode of life. *Potamonautes lividus* does not burrow to the same depths as *P. flavusjo* sp. nov., or *P. calcaratus*. All three of these species are ecologically distinct, with *P. flavusjo* sp. nov., being exclusively associated with peat soils in vlei areas on the Mpumalanga Highveld where it occurs under grass and reed banks. Burrows of *P. flavusjo* sp. nov., are generally straight and vertical, and typically up to 1 m into the peat soil, however the burrow depth is dependent on the depth of the water table (Daniels pers. obs). Burrows always have a single opening used for both entry and exit, with the crab generally occupying a small round chamber at the bottom of the burrow that is full of freshwater (pH of 7.38 –

near neutral, conductivity 103µS/cm, temperature of the water at bottom of the burrow 14.6°C, recorded only at the Iona farm sample site in the district of Chrissiesmeer). In contrast *P. lividus* forms U shaped burrows, < 30 cm deep (Gouws pers. comm) and is endemic to hydromorphic peat swamp forest areas (comprised of *Barringtonia*, *Ficus* and *Syzygium* trees) in north-eastern KwaZulu-Natal (Gouws *et al*., 2001). However, more recently a population of *P. lividus* was discovered at Dwessa forest in the Eastern Cape province of South Africa, approximately 750 km from its previously known distribution range in KwaZulu-Natal (Daniels pers. obs). Unpublished mtDNA sequence data reveals that the Eastern Cape and KwaZulu-Natal specimens are genetically nearly identical (Daniels pers. obs). *Potamonautes lividus* has also been collected under decaying logs of wood in forested areas, particularly following episodes of heavy rains (Daniels pers. obs). *Potamonautes calcaratus* is exclusive to the Kruger National Park in South Africa, although the species is also present in southern Mozambique and Zimbabwe (Reed and Cumberlidge, 2004). *Potamonautes calcaratus* can typically be found around ephemeral pans where they burrow into the banks of the pans. A single burrow opening is present in this species and the burrows can extend deep into the soil around the ephemeral pans.

Etymology: The name *P. flavusjo* sp. nov., is an arbitrary combination of two aspects. The species name comprises 'flavus' is Latin for yellow, a reference to the distinct canary yellow post frontal crest margin (Fig. 6) while 'jo' is added in honour of Dr Johan Engelbrecht, recently deceased, for his unwavering dedication and commitment to freshwater research in the Mpumalanga Province of South Africa and for sending the authors the first female specimen of the species. The name is used as a noun in apposition.

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Table 1 Substitution models and for each of the three mtDNA loci (12S rRNA, 16S rRNA and COI).

Gene fragment	No. of base pairs	Model	Base pair frequencies	Rate matrix	G distribution shape parameter	Proportion of invariable sites (I)
12S rRNA	316	K81uf + I + G	A = 40.51% C = 06.16% G = 15.62% T = 37.71%	R (a) [AC] = 1.00 R (b) [AG] = 6.15 R (c) [AT] = 0.53 R (d) [CG] = 0.53 R (e) [CT] = 6.15 R (f) [GT] = 1.00	0.84	0.41
16S rRNA	362	TIM + I + G	A = 38.48% C = 07.36% G = 13.78% T = 40.38%	R(a) [AC] = 1.00 R(b) [AG] =11.14 R(c) [AT] = 1.99 R(d) [CG] = 1.99 R(e) [CT] = 6.48 R(f) [GT] = 1.00	0.43	0.21
COI	600	GTR +I + G	A = 37.96% C = 15.24% G = 17.65% T = 29.16%	R(a) [AC] = 0.17 R(b) [AG] = 9.14 R(c) [AT] = 1.01 R(d) [CG] = 0.39 R(e) [CT] = 2.10 R(f) [GT] = 1.00	1.01	0.50

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Table 2 *Potamonautes bellarussus* sp. nov. measurements (in mm) of the holotype and ranges for additional material examined.

Variable	Abbreviation	Holotype	Males	Females
Carapace length	CL	39.29	22.91-33.31	18.07-35.73
Carapace width at widest point	CWW	58.42	32.71-47.98	25.18-48.64
Carapace posterior margin	CWP	17.54	12.32-16.73	9.75-18.71
Frontal width	FW	19.90	10.83-15.37	8.29.16.76
Distance between postfrontal crest and anterior margin	PFCD	6.01	3.03-4.16	2.59-4.20
Carapace height	CH	19.03	10.57-15.22	8.19-16.82
Major cheliped propodus length	MCPL	49.79	19.66-21.10	6.30-21.98
Pereopod 2, merus length	P2ML	22.40	13.63-19.64	10.21-18.85
Pereopod 2, merus width	P2MW	8.47	5.15-7.84	3.92-7.72
Pereopod 5, merus length	P5ML	18.25	11.53-19.01	10.58-18.85
Pereopod 5, merus width	P5MW	6.24	4.46-7.79	4.10-8.02

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Table 3 *Potamonautes flavusjo* sp. nov. Measurements (in mm) of the holotype and ranges for additional material examined.

Variable	Abbreviation	Holotype	Males	Females
Carapace length	CL	24.02	15.67-20.89	14.96-23.74
Carapace width at widest point	CWW	34.75	21.74-29.24	20.89-33.68
Carapace posterior margin	CWP	12.31	8.16-10.53	8.62-13.39
Frontal width	FD	14.20	9.67-12.75	8.49-14.32
Distance between postfrontal crest and anterior margin	PFCD	4.13	2.64-3.21	2.68-4.23
Carapace height	CH	14.87	9.05-12.70	9.52-14.78
Major cheliped propodus length	MCPL	22.86	12.35-18.70	11.91-20.79
Pereopod 2, merus length	P2ML	11.82	8.27-11.09	8.08-12.23
Pereopod 2, merus width	P2MW	4.50	3.30-4.78	3.67-5.20
Pereopod 5, merus length	P5ML	11.56	8.30-10.23	8.76-12.11
Pereopod 5, merus width	P5MW	4.47	4.28-4.43	3.80-4.97

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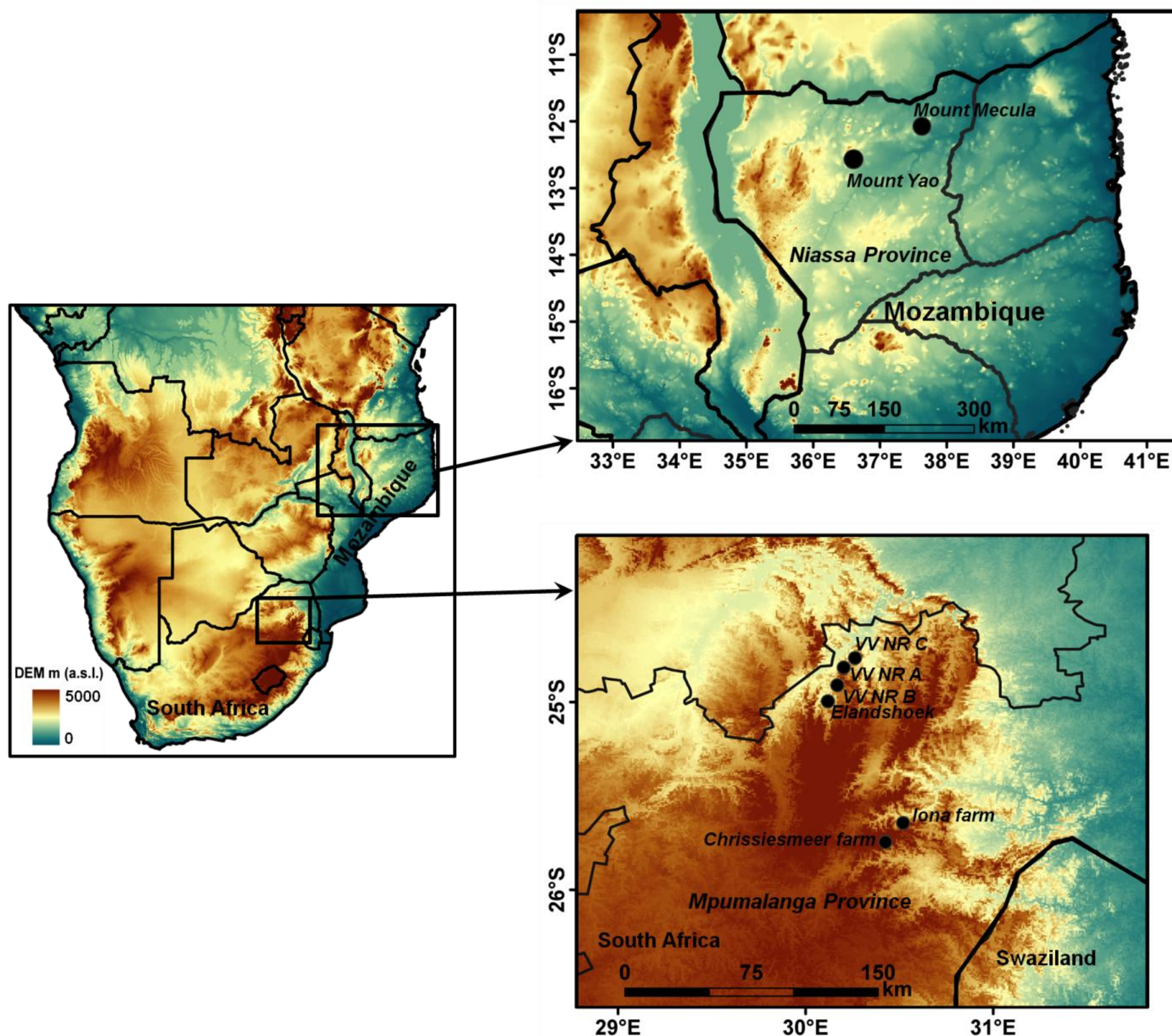

Figure 1 A map showing the two samples sites, Mounts Mecula and Yao in the Niassa Province, Mozambique and the six Highveld samples sites (Verloren Vallei Nature Reserve (VV NR: A, B and C), Elandshoek, Iona and Chrissiesmeer Guest farms in the Mpumalanga Province of South Africa where freshwater crabs were collected. The new Highveld species was collected sympatrically with *Potamonautes sidneyi* at four sample sites, Verloren Vallei Nature Reserve (NR) (sites B and C) and at the farm Iona and Miss Chrissie's Country House Guest farm, Chrissiesmeer.

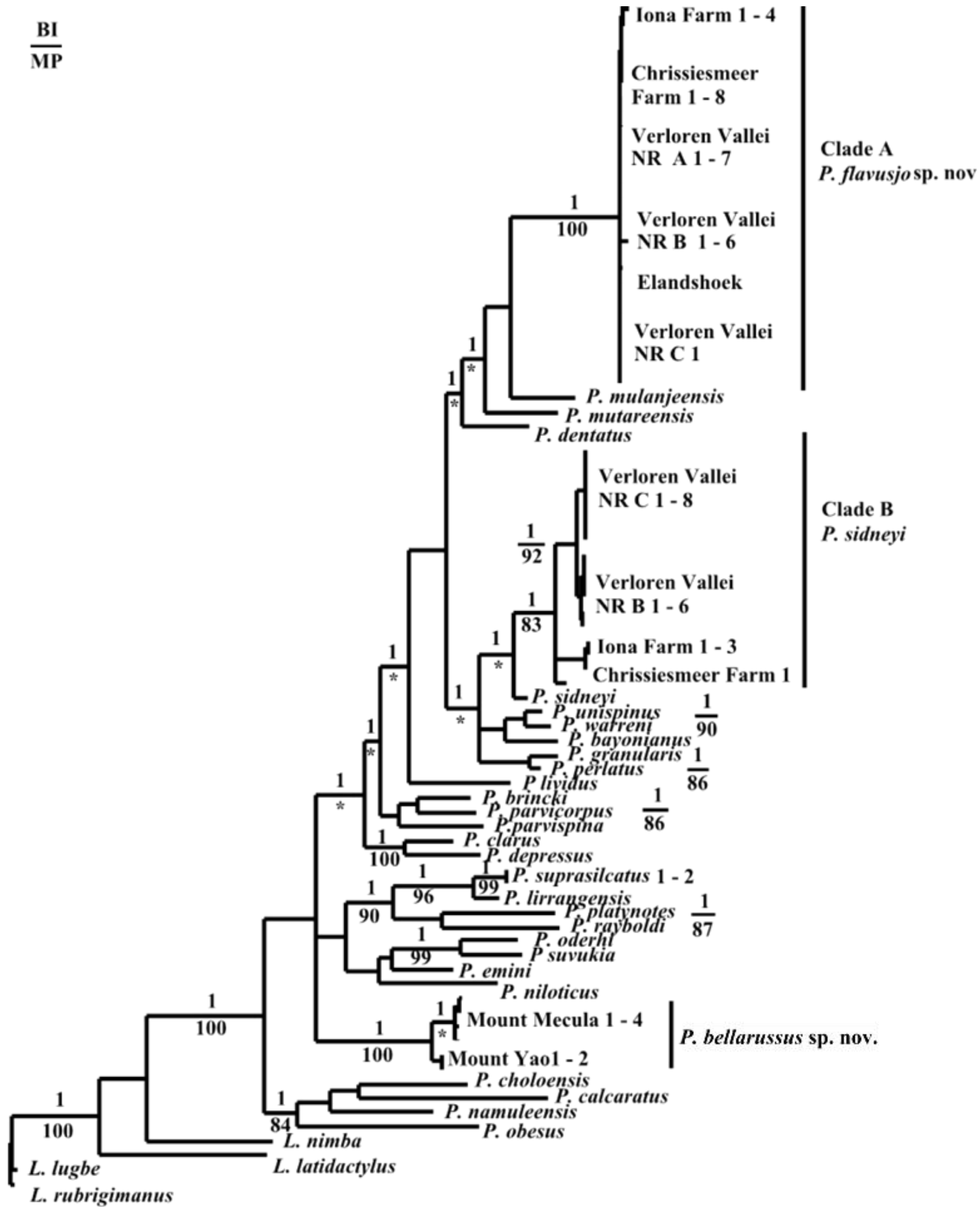

Figure 2 Combined Bayesian tree topology derived from the total evidence mtDNA data set for the three loci (12S rRNA, 16S rRNA, and COI). Values above the node represent the bootstrap values (%) for the nodes, while values below the node represent the posterior probability value (p*P*). Only bootstrap values >75% are shown, and posterior probability values of 1.00 are shown, and * indicates nodes that were not statistically supported.

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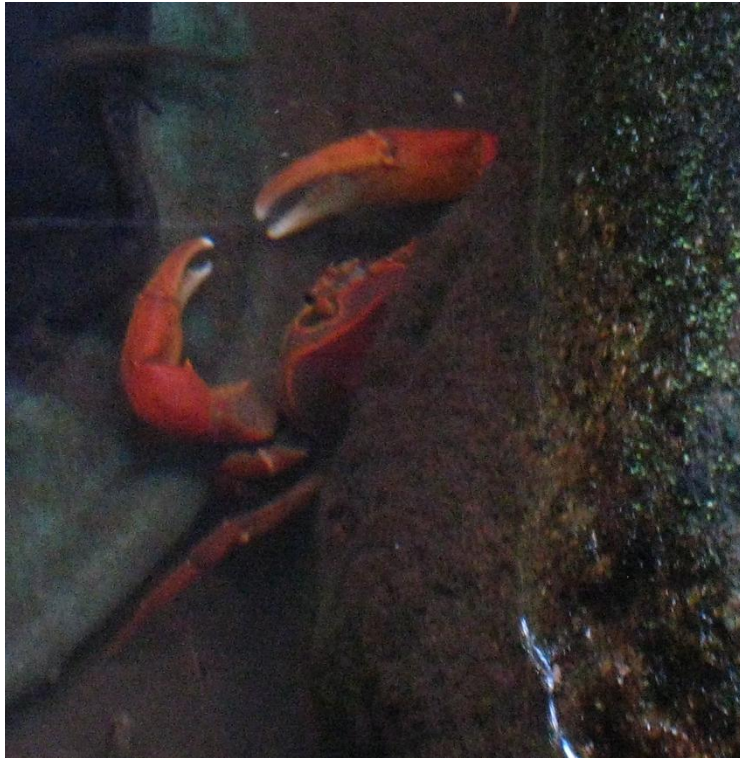

Figure 3 *Potamonautes bellarussus* sp. nov., female from the Mecula mountains in the Niassa province, Mozambique showing the bright red colour of the freshwater crab while alive.

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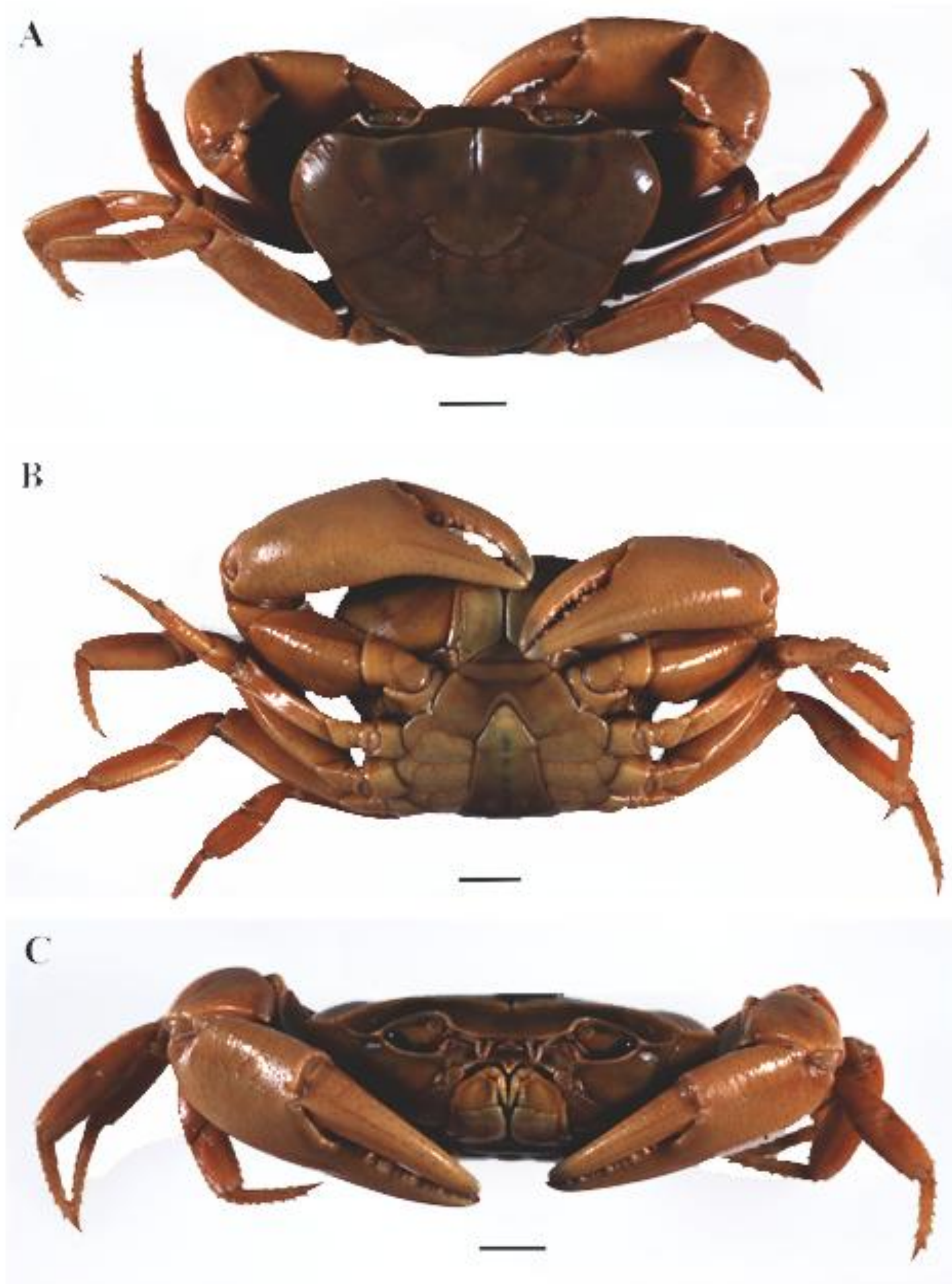

Figure 4 *Potamonautes bellarussus* sp. nov. male holotype (CL = 39.29 mm from the Yao mountains, 1045 m altitude above sea level (a.s.l.), 12º 27' 276" S, 36º 32' 260" E, Niassa Province, Mozambique (SAM A48212). A, whole animal dorsal aspect; B, whole animal ventral aspect; C, cephalothorax, frontal aspect. Scale line represents 10 mm.

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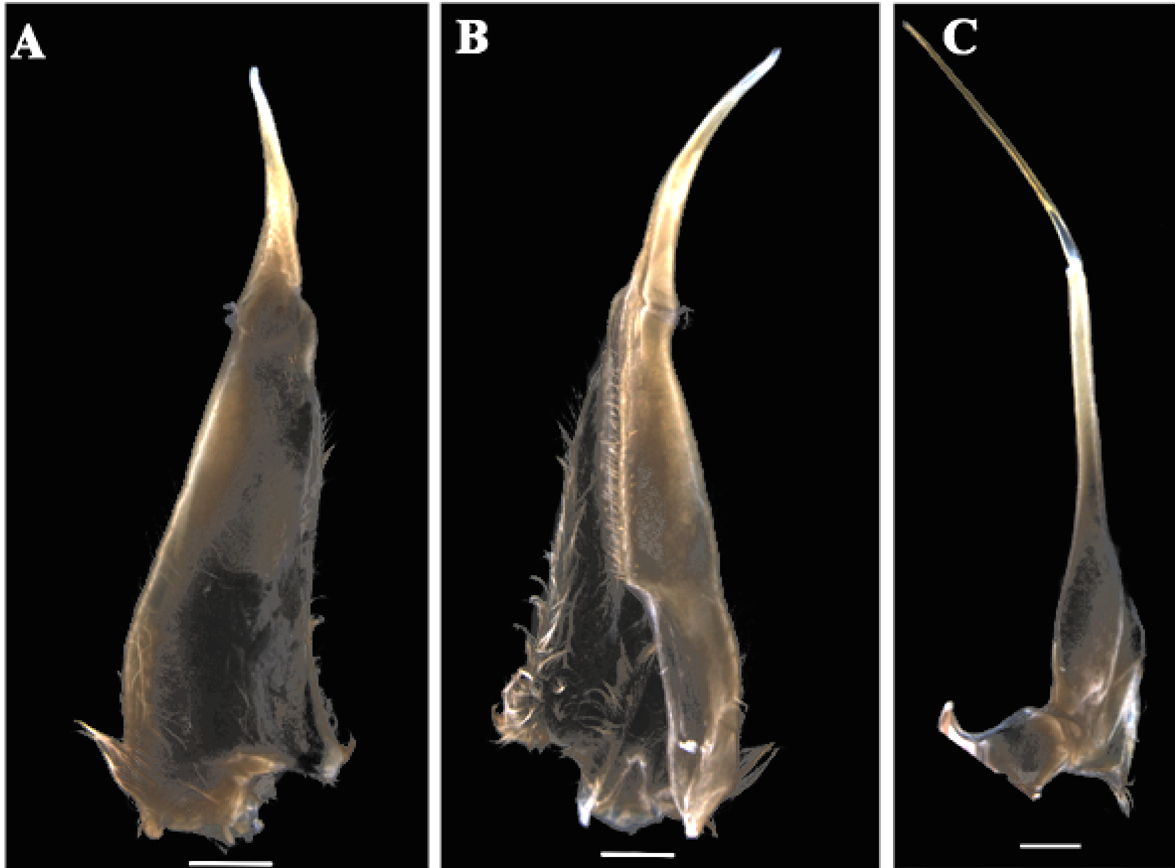

Figure 5 *Potamonautes bellarussus* sp. nov. male holotype from the Yao mountains, Niassa Province, Mozambique (SAM A48212). A, left gonopod 1, anterior view; B, left gonopod 1 posterior view; C, left gonopod 2 anterior view. The scale bar represents 1 mm.

Figure 6 Live image of a female *Potamonautes flavusjo* sp. nov. from Elandshoek, 1987 m above (SAM A48209), Mpumalanga Province of South Africa demonstrating the bright yellow post frontal crest margin. Photo taken by Gerhard Diedericks.

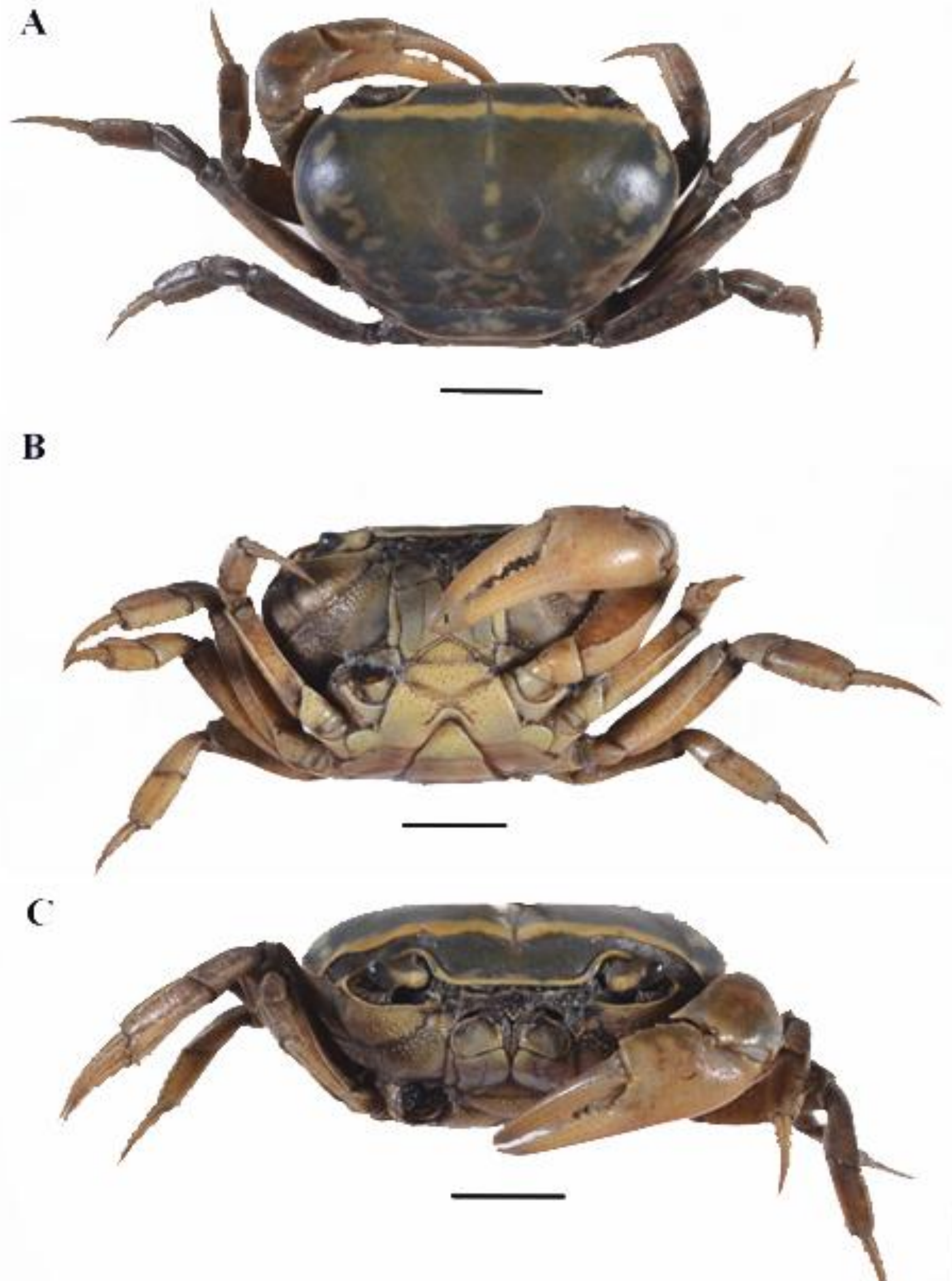

Figure 7 *Potamonautes flavusjo* sp. nov. male holotype (CL = 24.02 mm) Verloren Vallei Nature Reserve (B), 2000 m a.s.l., 25º 20’ 333” S, 30º 07’ 546” E, Mpumalanga Province of South Africa (SAM A48203). A, whole animal dorsal aspect; B, whole animal ventral aspect; C, cephalothorax, frontal aspect. Scale line represents 10 mm. The right cheliped was broken off prior to capture.

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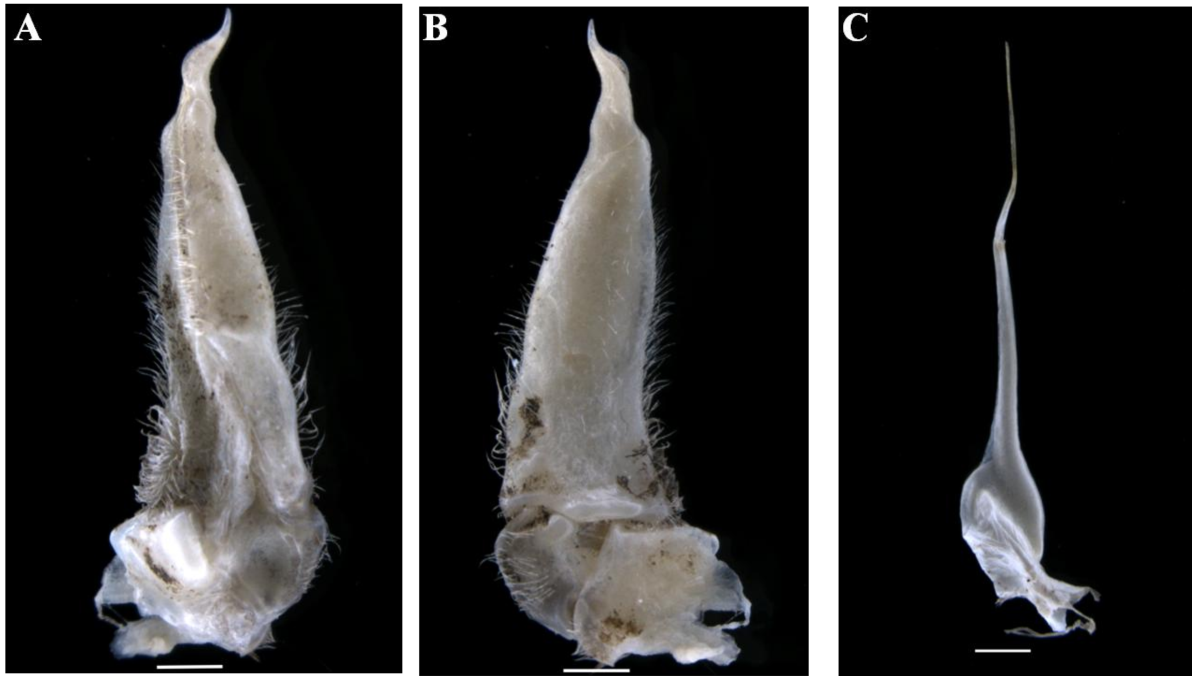

Figure 8 *Potamonautes flavusjo* sp. nov. male holotype, Verloren Vallei Nature Reserve (B), Mpumalanga Province of South Africa (SAM A48203). A, left gonopod 1, anterior view; B, left gonopod 1 posterior view; C, left gonopod 2 anterior view. The scale bar represents 1 mm.

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CHAPTER 6

BIOGEOGRAPHY AND PHYLOGENETICS OF THE AFROTROPICAL FRESHWATER CRAB FAUNA (BRACHYURA: DECAPODA: POTAMONAUTIDAE) EXPLORED WITH DENSER TAXONOMIC SAMPLING*

ABSTRACT

The freshwater crab superfamily Potamonautidae (Crustacea: Decapoda: Brachyura) is one of five freshwater crab families and is confined to the Afrotropical region, but phylogenetic relationships, particularly at the sub-family level are uncertain. In this study, I used one nuDNA (histone 3) and three mtDNA (12S rRNA, 16S rRNA, and COI) loci to examine the phylogenetic relationships between 84 Afrotropical freshwater crab species (representing 16 of 18 described genera). I used the mutation rates of mtDNA in BEAST to estimate the divergence times and to infer the evolutionary histories of the Afrotropical freshwater crab fauna. I also reconstructed their ancestral ranges using the Bayesian binary method (BBM). *Potamonautes*, the most species rich genus, was paraphyletic with *Platythelphusa* and comprised several distinct clades. While the isolation and speciation of *Platythelphusa* is associated with the isolation of an ancestral freshwater crab species that invaded Lake Tanganyika in East Africa, the occurrence of Potamonautidae on Madagascar and the Seychelles is linked to transoceanic dispersal. The results suggest that the Afrotropical Potamonautidae diverged in the late Cretaceous, approximately 107.57 Mya. Cladogenesis within this family occurred in Tertiary, which was associated with major uplift and rifting events on continental Africa, with southern Africa being the most recently diverged. The BBM suggested a West / East African ancestral range for the family, with 30 dispersal- and 15 vicariance events, and the West African genera (*Liberonautes*, *Sudanonautes*, and *Potamonemus*) formed the basal / ancestral group. A close phylogenetic relationship between geographically disconnected clades (i.e. East and West Africa) was observed, which suggests ancient connections between East and West African freshwater crabs. For other taxa, close East / West phylogenetic relationships have been attributed to a continuous forest belt that stretched from the Eastern Arc Mountains to the West-Central African Guineo-Congolian rainforest. For freshwater crabs, the contraction of forests, and subsequent drainage contractions, during the Tertiary may thus have resulted in relictual phylogenetic relationships rather than recent divergence between the East and West African

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freshwater crab fauna. The Potamonautidae need revision at the sub-family level in order to stabilize the classification of the group.

INTRODUCTION

Biogeography is primarily concerned with obtaining evolutionary relationships that are rooted in phylogenies of organisms inhabiting a particular geographical region by inferring their patterns of dispersal and speciation (Wiley, 1988; Lowe-McConnell, 1987; Morrone & Cristi, 1995; Lévêque, 1997; Santini & Winterbottom, 2002; Domínguez-Domínguez *et al.*, 2006). Observed biogeographical patterns are subsequently also a representation of the processes that shape the dispersal of species at various spatial and temporal scales (Wiens & Donoghue, 2004).

A range of hypotheses have been proposed to explain the biogeography of organisms, and have focussed on dispersal (centre of origin) and vicariance theories (Wiley, 1988; Morrone & Cristi, 1995; Wiens & Donoghue, 2004; Heads, 2010). The dispersal or centre of origin hypothesis states that disjunct distribution patterns of species among related taxa may exhibit the observed patterns because a common ancestor originally occurred in a specific area and later dispersed to areas where descendants occur at present (Wiley, 1988; Morrone & Cristi, 1995). The vicariance hypothesis, on the other hand, states that a common ancestor of related taxa was originally widespread, and its descendants diverged following habitat fragmentation (Wiley, 1988; Morrone & Cristi, 1995), resulting in allopatric speciation. Therefore, different monophyletic groups of taxa that display similar biogeographic patterns are likely to share the same biogeographic history (Donoghue & Moore, 2003; Wiens & Donoghue, 2004; Ree *et al.*, 2005). In order to make inferences regarding the spatial and temporal phylogenetic histories of monophyletic groups of taxa, the evidence of vicariance, dispersal, and paleogeographical events has to be taken into account (Waters *et al.*, 2001; Ree *et al.*, 2005), with the expectation that closely related species would likely replace each other in space. However, a fundamental question that remains is when ancestor species colonized their environments in the first place.

Islands have often been the basis for numerous evolutionary biogeography studies because they represent the most obvious natural examples of habitat discontinuity and / or fragmentation. However, inland aquatic freshwater systems (e.g. rivers and lakes) represent interesting naturally fragmented habitats for examining hypotheses of historical biogeography. Owing to their patchiness, inland aquatic environments can also be considered

as islands with their own environmental characteristics. The distribution of freshwater taxa within biogeographical regions are further divided into isolated groups by, for instance, large drainage river systems with hydrographical networks that form isolated freshwater systems (e.g. Amazon and Congo River basins; Gascon *et al*., 1998; Hughes *et al*., 1992; Belliard *et al*., 1997; Fraser & Keddy, 2005). These hydrographical networks ultimately serve as barriers that limit species distribution and faunal exchange between drainages, shaping patterns of diversification (Gascon *et al*., 2002; Cook *et al*., 2002) as well as intraspecific genetic delineation (Nicolas *et al*., 2011). This has been demonstrated for the distribution of terrestrial and amphibious vertebrates (Gascon *et al*., 1998, 2002; Nicolas *et al*., 2011) and even birds (Hayes & Sewlal, 2004). Similarly, isolated inland aquatic habitats can be effective barriers restricting gene flow between freshwater taxa promoting cladogenesis (e.g. Meffe & Vrijenhoek, 1988; Cook *et al*., 2002; Waters *et al*., 2001; Hughes *et al*., 2004; Cook *et al*., 2006; Dominguez-Dominguez *et al*., 2006; Hubert & Renno, 2006; Mock *et al*., 2010). As such, some populations have as a result evolved independently in different freshwater systems with significant degrees of endemism, with some species highly adapted to their respective environments as well as to the structural heterogeneity of the habitats, which are two of the major ecological factors that contribute to the existence and sustainability of species through space and time (Lévêque, 1997).

Thus, owing to their generally limited dispersal abilities, relatively low fecundity, intolerance to desiccation and saline habitats, as well as restriction by ecosystem microhabitat requirements (Lowe-McConnell, 1987; Lévêque, 1997; Thieme *et al*., 2005), the vast majority of primary freshwater taxa are endemics. Investigations of the historical biogeography of freshwater taxa have revealed likely historical connectivities between these habitats, especially in large river drainage systems. Therefore, timing divergence events is useful for the inference of species diversity and patterns of diversification (Genner *et al*., 2007). Moreover, understanding the evolution and distribution patterns freshwater taxa can provide important insights into the histories of the isolation, interconnection processes, and the development of hydrographic boundaries. Nonetheless, owing to the lack of reliable molecular clock estimates as well as fossils for numerous freshwater taxa, the geological events associated with the contemporary distributions of these taxa are poorly resolved (Genner *et al*., 2007). Besides studies on fishes (e.g. Lévêque, 1997; Skelton, 2001; Salzburger *et al*., 2002, 2005; Kocher, 2004; Thieme *et al*., 2005; Genner *et al*., 2007; Koblmüller *et al*., 2008; Pinton *et al*., 2013), few large-scale biogeographic studies have

focused on other freshwater taxa such as crabs to test their utility as biogeographic indicators on continental Africa (e.g. Daniels *et al*., 2006a).

In recent years, the Afrotropical freshwater crabs (Potamonautidae) have received extensive attention in studies involving molecular phylogenetics, and most of these studies are of South African freshwater crabs (Daniels *et al*., 1999; 2001; 2002, 2003; Daniels, 2003; Daniels *et al*., 2006b; Phiri & Daniels, 2014), with some studies also conducted in East African great lake systems (e.g. Marijnissen *et al*., 2006). However, there is still only one comprehensive study (Daniels *et al*., 2006a) that has attempted to verify phylogenetic relationships within the Afrotropical freshwater crab fauna. While Daniels *et al*. (2006a) also placed the Afrotropical freshwater crabs within the phylogeny of the global freshwater crabs, their study suffered from limited taxonomic sampling. Currently, the Afrotropical region represents roughly 10% of the global species diversity of freshwater crabs, but this is likely to be a significant underestimate, especially with the recent discovery of multiple novel and cryptic lineages within presumably widely distributed species (Daniels *et al*., 2003; Daniels & Bayliss, 2012; Cumberlidge & Daniels, 2014; Phiri & Daniels, 2014; Chapter 3, this thesis). As a consequence of the paucity of studies concentrating on phylogenetic relationships of freshwater crabs, the classification and assignment of the Afrotropical freshwater crab fauna has remained reasonably slow.

There are two recent taxonomic revisions of the Afrotropical freshwater crab fauna at higher taxonomic levels (Cumberlidge *et al*., 2008; De Grave *et al*., 2009). Cumberlidge *et al*. (2008) based their classifications on phylogenetic results from three studies, i.e. Spears *et al*. (2004), Daniels *et al*. (2006a), and Klaus *et al*. (2006). The classification by De Grave *et al*. (2009) was loosely based on Martin & Davis (2001), but has some discrepancies such as the dubious placement of some genera in the sub-families, i.e. *Foza* and *Louisea* (Table 1). De Grave *et al*. (2009) placed of *Foza* in the Potamonautinae, with incorrect taxonomic authorities for the genus and called it *Foza* Dai & Bo, 1994, whereas this genus was established by Reed & Cumberlidge, 2006). Moreover, Grave *et al*. (2009) placed *Louisea* in the Hydrothelphusinae following Ng *et al*. (2008), who recognized two subfamilies for the Potamonautidae: Potamonautinae and Hydrothelphusinae. Therefore, these recent sub-family level taxonomic classifications (Cumberlidge *et al*., 2008; De Grave *et al*., 2009) highlight uncertainties regarding the generic placements within sub-families (Table 1) mainly because there is disagreement on the number of sub-families within the Potamonautidae – two sub-families (Potamonautinae and Deckeniinae; Cumberlidge *et al*., 2008) or three sub-families (Potamonautinae, Deckeniinae, Hydrothelphusinae; De Grave *et al*., 2009).

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There are various contrasting hypotheses regarding the historical biogeography of freshwater crabs in the Afrotropical region. Bott (1972) hypothesized that the widespread distribution of the Potamonautidae took place during the Tertiary, where a marine crab ancestral population living in the Tethys Sea, north-west of present day Africa, entered the inland waters of the continent. The continent-wide dispersal of the Potamonautidae subsequently led to the development of endemic sub-groups of African freshwater crabs, and presumably to the 15 sub-genera of *Potamonautes* proposed by Bott (1955). Kensley (1981) suggested that *Potamonautes* originated in North Africa and reached southern Africa following a migration southwards via the river systems and lakes of the Rift Valley. This genus then formed a number of geographically isolated species groups in the major river basins of southern Africa, roughly corresponding to some of Bott's (1955) sub-genera. Daniels *et al*. (2002) proposed that speciation of southern African freshwater crabs, resulting from isolation of drainage systems, reflects the impact of past climatic change on river drainage system isolation.

These dispersal hypotheses would be supported by a phylogenetic pattern that reflects the history of the main riverine drainage systems of Africa. However, many long-established freshwater bioregions are delimited according to freshwater fish distribution (e.g. Skelton, 2001; Lévêque, 1997) without the consideration of macro-invertebrates, such as freshwater crabs. It is clear however that the hydrographical networks of continental Africa underwent extensive modifications as a result of successive geological uplifts and collapses from the Miocene onwards (John, 1986). By the Pliocene, many rifting and uplift episodes occurred in East Africa and various other drainage systems were transformed or became landlocked. Recently, Linder *et al*. (2012) used multivariate methods to statistically partition sub-Saharan Africa into seven bioregions based on the regionalization of vascular plants and vertebrates (mammals, birds, amphibians, reptiles), i.e. Congolian, Zambezian, Southern African, Sudanian, Somalian, Ethiopian, and Saharan regions (see Results section). Linder *et al.* (2012) also provided a phenogram representing relationships between taxa occurring in the defined bioregions, but excluded invertebrate freshwater taxa.

The aim of this study was to use molecular techniques to re-examine the phylogenetic relationships among Afrotropical freshwater crab fauna within the Potamonautidae in order to obtain a better resolution on their phylogeny. I explored their biogeographical affinities to explore hypotheses of origin and dispersal using a Bayesian binary ancestral reconstruction method in conjunction with divergence time estimations. Specifically, I attempt to determine the causative biogeographical mechanisms that shaped the phylogenetic relationships within

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the Potamonautidae by conducting a robust phylogenetic study based on extensive taxonomic sampling.

MATERIALS AND METHODS

STUDY AREA AND SPECIES

The Afrotropical region is defined as the area in sub-Saharan Africa, below the Sahara Desert, and includes the African mainland as well as the Indian Ocean islands of Madagascar, the Seychelles, and Socotra, which are fragments of Gondwana (Fig. 1). The previous study by Daniels *et al*. (2006a) included 13 genera, comprising 40 species. In this study I included 84 species within the Potamonautidae, representing 16 of the 18 genera that occur in the Afrotropical region (Table 2). *Louisea* and *Erimetopus* are the only two missing genera because I could not obtain any usable sequence data from the museum specimens received. Freshwater crabs from Socotra belong to the Potamidae (North African family) and are closely related to two Asian genera *Geothelphusa* and *Johora* (Daniels *et al*., 2006a). In the present study, I used *Johora tiamanemsis*, *Socotrapotamon socotrensis*, and *Potamon fluviatile* (Europe) as outgroups. The Afrotropical genera included in this study were as follows: *Afrithelphusa* (n = 1), *Boreathelphusa* (n = 1), *Deckenia* (n = 2), *Foza* (n = 1), *Globonautes* (n = 1), *Hydrothelphusa* (n = 5), *Liberonautes* (n = 6), *Madagapotamon* (n = 1), *Malagasya* (n = 2), *Marojejy* (n = 1), *Potamonautes* (n = 39), *Potamonemus* (n = 3), *Platythelphusa* (n = 8), *Seychellum* (n = 3), *Skelosphusa* (n = 2), and *Sudanonautes* (n = 2). The different freshwater crab genera were identified following the morphological descriptions compiled by Bott (1955) and Cumberlidge (1999) for the Potamonautinae (excluding *Platythelphusa*), as well as Cumberlidge & Sternberg (2002) for the Hydrothelphusini.

MOLECULAR TECHNIQUES AND SEQUENCING

DNA was extracted from either pereopod muscle or gill tissue samples provided by the various museums or from personal collections (Table 2); one specimen per species was used. I extracted DNA from between 0.5 and 1 g of tissue from each specimen (Daniels, 2003; Daniels *et al*., 2006a) using a QIAgen DNAeasy kit following the manufacturers' protocol. DNA was then stored at -20 °C until required for polymerase chain reaction (PCR) (Daniels, 2003; Daniels *et al*., 2006a).

Before use in PCR, a 1 µL:19 µL (DNA: millipore water). I amplified three mitochondrial partial gene fragments (mtDNA): 12S rRNA (12Sai 5'-AAA CTA GGA TTA

GAT ACC CTA TTA T-3’ and 12Sb 5’-GAG AGT GAC GGG CGA TGT GT-3’) (Kocher *et al*., 1989), 16S rRNA (16SA 5’-ACT TGA TAT ATA ATT AAA GGG CCG-3’ and16SB 5’-CTG GCG CCG CTC TGA ACT CAA ATC-3’) (Palumbi *et al*., 1991), and cytochrome oxidase I (COI; LCOI-1490 5’-GGT CAA CAAA TCA TAAA GAT ATTG-3’ and HCOI-2198 5’-TAAA CTT CAG GGT GAC CAAA AAA TCA-3’) (Folmer *et al*., 1994). I also included one nuclear DNA (nuDNA) genetic marker, histone three (H3; H3AF 5’-ATG GCT CGT ACC AAG CAG ACVGC-3 and H3AR 5’-ATA TCC TTR GGC ATR ATR GTG AC-3’) (Colgan *et al*., 1998). Daniels *et al*. (2006a) used these markers in their study on the evolution of Afrotropical freshwater crabs. I also included all the *Platythelphusa* species from GenBank (see Table 2; Marijnissen *et al*., 2006), as well as all subsequent genetic studies on the Afrotropical freshwater crabs (Daniels 2011; Daniels & Bayliss, 2012; Phiri & Daniels, 2013; Cumberlidge & Daniels, 2014; Phiri & Daniels, 2014). For some specimens I could not obtain usable sequences for some markers (see Table 2), so those were coded as missing data during phylogenetic reconstructions.

For PCR, I used a 25 µL reaction solution for each sample: millipore water, 25 mM $MgCl_2$, 10X Mg^{2+} free buffer, 10 mM dNTP solution, 10 mM forward and reverse genetic marker primers, 0.1 µL U Taq polymerase, and 2.5 µL of the 1:19 µL template DNA dilution. For 12S rRNA and 16S rRNA the following PCR conditions were used: 95°C (5 min.), [95°C (30 sec), 50°C (40 sec.), 72°C (1 min.)] for 36 cycles; for COI: 94°C (4 min.), [94°C (30 sec.), 42°C (40 sec.), 72°C (45 sec.)] for 36 cycles; and for H3: 95 °C (5 min.), 95 °C (30 sec.), 48 to 46 °C (40 sec.), 72 °C (1 min). The final extension for all PCR conditions was, 72°C (10 min.). PCR products were electrophoresed (4 hours in a 1% agarose gel containing ethidium bromide). DNA fragments were then purified with the BioFlux purification kit (Bioer Technology Co., Ltd), after which they were sent to Macrogen for sequencing (Macrogen Inc. Amsterdam, The Netherlands; http://www.macrogen.com).

Sequences were checked for ambiguities and aligned with MUSCLE Multiple Alignment as executed in MEGA5 v. 2.2 (Tamura *et al*., 2011). The COI (protein-coding genetic marker) sequences were translated to amino acids and checked for stop codons using EMBOSS-Transeq (http://www.ebi.ac.uk/emboss/transeq/).

PHYLOGENETIC RECONSTRUCTIONS

I reconstructed the Afrotropical freshwater crab phylogeny using maximum likelihood (ML), and Bayesian inference (BI). Analyses were conducted separately to confirm topology

congruence. The analyses were then run for the combined dataset (the four loci). ML was executed in MEGA5 v. 2.2 (Tamura *et al*., 2011). Bootstrap trees were inferred from 10 x 10^3 replicates and the Tamura 3-parameter model with gamma-distributed rate heterogeneity (T92 + G) was used as the substitution model (obtained in MEGA5 v. 2.2; Nei & Kumar, 2000; Tamura *et al*., 2011). The ML tree was obtained using the Close-Neighbour-Interchange algorithm (Nei & Kumar, 2000) and the initial trees were obtained with the random addition of sequences (ten replicates). Branches for partitions reproduced with <50% bootstrap replicates were collapsed.

For the BI analyses, the best-fit models for individual genes were obtained by using jModelTest v. 2.1.3 (Posada, 2008) and the best-fit maximum likelihood scores were chosen using Akaike's Information Criterion (AIC) (Akaike, 1973). The Bayesian phylogenetic reconstruction was executed in MrBayes v. 3.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003; Ronquist *et al*., 2012). Consensus trees were constructed in a partitioned analysis with four Markov Chain Monte Carlo (MCMC) simulations run for 5 x 10^6 generations, and each chain starting from a random tree. Sampling from the chain was conducted every 5 x 10^3 generations. Convergence was reached when the standard deviation of split frequencies was <0.01 and effective sample size values (ESS) >100 (Ronquist & Huelsenbeck, 2003; Ronquist *et al*., 2012). A 50% majority rule tree was retained after a 25% burn-in. The consensus tree was viewed and edited in FigTree v. 1.4 (Drummond *et al*., 2009).

The best tree topology from each of the phylogenetic reconstruction methods was chosen and drawn to scale. Nodes were considered to be statistically supported when ML bootstrap values were ≥75% and the BI posterior probabilities (p*P*) ≥0.95.

Divergence time estimations

Divergence times for the combined dataset (12S rRNA, 16S rRNA, and COI) were estimated in BEAST v.2.0.2 (Drummond *et al*., 2002, 2009, 2012a, b). 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). I used mutation rates because there are no fossil records for the Afrotropical freshwater crab fauna (Daniels *et al*., 2006a; Daniels, 2011). There is no accepted mutation rate for either 12S rRNA or H3. For COI, the mean mutation rate used was 2.0 x 10^{-8} per Myr (± 6.40 x 10^{-9} – 1.42 x 10^{-8}, standard deviation (SD) = 3.059 x10^{-9}) and for 16S rRNA, 1.02 x 10^{-8} per Myr (± 1.40 x 10^{-8} – 2.60 x 10^{-8}, SD = 1.94 x 10^{-9}) per Myr. These

rates are widely accepted for estimating divergence time for decapods (Jesse *et al*., 2010; Daniels, 2011; Shih *et al*., 2011; Klaus *et al*., 2013). The mutation rates for 12S rRNA and H3 were estimated around the mutation rates of 16S rRNA and COI under a broad uniform substitution rate prior.

BEAUti v.2.0.2 (Drummond & Rambaut, 2007; Drummond *et al*., 2012a, b) was used for creating the BEAST input (xml) file. The substitution models (obtained in jModelTest) and clock rates were unlinked, the mtDNA trees (12S rRNA, 16S rRNA, and COI) were linked because they are linked in the mitochondria, while the nuDNA (H3) tree was unlinked from the mtDNA tree. The Yule speciation process was used together with an uncorrelated log-normal relaxed molecular clock model to infer divergence times with the incorporation of tree uncertainty in the Markov Chain Monte Carlo (MCMC) method (Heled & Drummond, 2012). Four MCMC chains were run for 200 x 10^6 iterations, with chains and trees sampled every 20 x 10^3 generations. The convergence of the four chains was verified by ESS >200 for each parameter in Tracer v. 1.5 (Drummond & Rambaut, 2007; Drummond *et al*., 2009). The resulting trees were combined (TreeAnnotator v. 2.0.2, part of the BEAST software package) and a maximum clade credibility tree was computed with mean heights after a 10% burn-in. FigTree v.1.4 was used to visualize and edit the tree (Drummond *et al*., 2009).

BIOGEOGRAPHY: ANCESTRAL RECONSTRUCTION

The Bayesian Binary Method (BBM) was used to infer the ancestral ranges of the Afrotropical Potamonautidae at key nodes. The distribution ranges were coded as follows: West Africa (A), Madagascar (B), East Africa (C), Central Africa (D), southern Africa (F), Seychelles (G), while E, H, and I were assigned to the outgroups. BBM is implemented in Reconstruct Ancestral States in Phylogenies (RASP v. 2.1b) (Yu *et al*., 2011). This method reconstructs ancestral areas derived from a sample of trees. Here, I used the divergence time estimation trees (10001 trees) obtained from the MCMC BEAST annotated tree to generate credibility support at each node and to account for alternative phylogenetic hypotheses. BBM averages the frequencies of ancestral ranges for each node across all trees and calculates nodal posterior probabilities (statistical support) of each ancestral range over a set of trees (Yu *et al*., 2011). MCMC chains were run for 5 x 10^6 generations, sampling every 1 x 10^3 generations, with a fixed JC + G (Jukes-Cantor + Gamma) and a null root distribution (Yu *et al*., 2011). Two concurrent analyses were conducted and a distance of less than 0.01 between runs was an indication of convergence. This recently developed method has been used to

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infer ancestral ranges for various taxa, including freshwater fish (Ali *et al*., 2013; Gao *et al*., 2013, Harris *et al*., 2013; Iverson *et al*., 2013; Lin & Hastings 2013). In addition, I also compared the phylogenetic reconstructions and divergence time estimations topologies with the ecoregions and topology (phenogram) proposed by Linder *et al*. (2012).

RESULTS

PHYLOGENETIC RECONSTRUCTIONS

The four loci that were sequenced (12S rRNA, 16S rRNA, COI, and H3) yielded a combined dataset comprising 1712 bp. Substitution model information and individual fragment lengths for each gene are summarised in Table 3. Phylogenetic reconstructions (ML and BI) showed that the Afrotropical Potamonautidae are a monophyletic group (ML = 95%, p*P* = 1.00) (Fig. 2). The sister relationship (ML = 94%, p*P* = 1.00) between *Sudanonautes* (West Africa) and *Potamonemus* (West Africa) was the most basal group (clade 1), followed by *Liberonautes* (West Africa), which also formed a monophyletic clade (clade 2). The next clade was a sister relationship (ML = 100%, p*P* = 1.00) between the monophyletic Malagasy freshwater crab fauna (ML = 100%, p*P* = 1.00) and the Seychelles / East Africa / West Africa (ML = 95%, p*P* = 1.00). *Seychellum* (ML = 100%, p*P* = 1.00) formed as sister relationship (ML = 97%, p*P* = 1.00) with the East African *Deckenia* (ML = 100%, p*P* = 1.00). This Seychelles / East Africa sub-clade was sister to the West African *Afrithelphusa* / *Globonautes* (ML = 100, p*P* = 1.00). *Potamonautes* formed a well-supported group (ML = 100, p*P* = 1.00), but was paraphyletic, with the monophyletic *Platythelphusa* (ML = 100, p*P* = 1.00). This paraphyletic relationship was only supported by BI (p*P* = 0.98, ML <75). East African *Potamonautes* species formed the basal group (ML = 99%, p*P* = 1.00; clade 4) for the continental Potamonautidae, followed by *Platythelphusa*, which was sister to *Potamonautes loveni* (East Africa) (ML = 95%, p*P* < 0.95; clade 5), with *Potamonautes kundondo* being the most basal species in the clade. The East African *Potamonautes* formed a monophyletic group (ML = 100%, p*P* = 1.00) (clade 6). Clade 7 was made up of four groups (ML <75%, p*P* <0.95): A = mountainous South African *Potamonautes* (ML = 95%, p*P* <0.95), B = four Angolan species, three of which are undescribed (ML = 91%, p*P* < 0.95), C = East and South African *Potamonautes* (ML <75%, p*P* < 0.95), and D = low-lying South African *Potamonautes* species (ML = 93%, p*P* < 0.95).

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DIVERGENCE TIME ESTIMATION

Divergence from the most recent common ancestor of the Potamonautidae occurred in the late Cretaceous, approximately 107.57 Mya (Fig. 3). The divergence time estimations topology differed slightly from the phylogenetic reconstructions. Though still basal, the West African Potamonautidae, *Sudanonautes* / *Potamonemus* and *Liberonautes* formed a sister relationship and diverged from the rest of the Potamonautidae 96.04 Mya (95% HPD: 78.53 – 109.33 Mya) – the first appearance of the Potamonautinae sub-family. *Liberonautes* split from the *Sudanonautes* / *Potamonemus* clade *c*. 95.60 Mya (95% HPD: 82.90 – 109.33 Mya), while *Sudanonautes* split from *Potamonemus c*. 74.04 Mya (95% HPD: 57.96 – 97.25 Mya). The Malagasy / Seychelles (including one East and two West African genera), i.e. the sub-family Deckeniinae, diverged from continental African Potamonautinae *c*. 77.83 Mya (95% HPD: 57.23 – 97.34 Mya). In the early Tertiary *c*. 62.60 Mya (Palaeocene) (95% HPD: 59.21 – 104.60 Mya), the Malagasy freshwater crab fauna (Hydrothelphusini tribe), diverged from the rest of the Deckeniinae. The Hydrothelphusini diverged into contemporary genera from 37.83 Mya (early Oligocene) (95% HPD: 31.07 – 83.06 Mya). Approximately 50.59 Mya (95% HPD: 44.95 – 98.18 Mya) (early Eocene), a common ancestor of the Deckeniini (Seychelles and East Africa) and the Globonautina (West Africa) diverged into these two tribes, where divergence within the Globonautina occurring in the early Miocene. The Seychelles split from the East African Deckeniini in the Oligocene (35.88 Mya, 95% HPD: 18.16 – 56.61 Mya). However, the descendants of this ancestor only diverged in the Pliocene (East Africa *c*. 9.5 Mya, 95% HPD: 4.75 – 13.81 Mya; and Seychelles *c*. 13.24 Mya, 95% HPD: 5.61 – 16.86 Mya).

The divergence of the rest of the continental Potamonautinae occurred *c*. 51.61 Mya (95% HPD: 39.07 – 75.06), which was around the time that the Deckeniinae split into the Deckeniini and Globonautina in the early Eocene. East African *Potamonautes* formed the basal group and appeared between 46.37 and 38.85 Mya (95% HPD: 21.59 – 71.11 Mya) from the mid-Eocene to early Oligocene. Also in the Eocene (42.22 Mya, 95% HPD: 28.41 – 66.42 Mya), the Southern African *Potamonautes* species appeared and split from the rest of the East African Potamonautinae. The East, West, and Central African Potamonatinae are made up of *Potamonautes* and *Platythelphusa*. Divergence within this group occurred in the Oligocene (34.53 Mya, HPD: 26.90 – 79.45 Mya), after which *Platythelphusa*, and its sister relationship with *Potamonautes loveni* (East Africa) occurred *c*. 35.37 Mya (95% HPD: 24.58 – 62.71). Divergence within *Platythelphusa* after splitting from its ancestor only occurred in

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the early Pliocene (*c*. 13.10 Mya, 95% HPD: 5.81 – 20.70 Mya). Approximately 20.61 Mya (95% HPD: 14.17 – 30.20 Mya) in the early Miocene, the West (*Potamonautes ecorssei*, Mali) and Central African (undescribed) species diverged from the rest of East Africa. The southern African *Potamonautes* diverged 32.95 Mya (95% HPD: 15.43 – 53.92 Mya), with the South African freshwater crab taxa forming a basal clade, diverging into different species in the Miocene from 22.86 Mya onwards (95% HPD: 11.75 – 46.18 Mya). Another Southern Africa / East Africa split occurred 23.25 Mya (95% HPD: 8.98 – 41.08 Mya), with another split (Southern Africa / West and Central Africa) occurring 24.73 Mya (95% HPD: 12.55 – 47.27 Mya). From there, West and Central Africa split from each other 20.55 Mya (95% HPD: 14.17 – 30.20 Mya).

BIOGEOGRAPHY: ANCESTRAL RECONSTRUCTION

The two concurrently run ancestral reconstructions analyses reached convergence (i.e. the distance between run 1 and run 2 was 0.0014). The ancestral reconstructions suggest a complex biogeographical history for the Potamonautidae. According to the BBM analysis, there were 30 dispersal, 15 vicariance, and no extinction events. Only the ancestral ranges of key nodes are presented in Fig. 4. The ancestral ranges were either or a combination of West Africa (A), Madagascar (B), East Africa (C), Central Africa (D), southern Africa (F), Seychelles (G). Distributions E, H, and I were assigned to the outgroups. BBM suggested three possible ancestral ranges for Potamonautidae, i.e. East (C), West (A), and / or AC, with the frequency of occurrence being 48.31%, 24.80%, and 16.11%, respectively, and p*P* = 0.39 (node 1, Fig. 4). The remaining 10.77% was suggested to be of unknown or mixed origins (MO). For this node, there was one vicariance and two dispersal events (RASP route (RR): C->AC->A|C). There was no dispersal or vicariance for the *Liberonautes* and *Sudanonautes* / *Potamonemus* split (RR: A->A^A->A|A; A, 99.46%, MO, 0.54%; p*P* = 0.99; node 2). There was one vicariance and two dispersal events for the Hydrothelphusini and Deckeniini / Globonautina split (RR: A->AB->A|B; A (79.38%), AB (5.99%), B (5.92%), MO (8.68%); p*P* = 0.68; node 4). The split of the Globonautina from the Deckeniini comprised one vicariance and two dispersal events (RR: A->AC->A|C; A (90.73%), MO (9.28%), p*P* = 0.48; node 5). Within the Deckeniini, the split of the Seychelles freshwater crabs from *Deckenia* had one vicariance and two dispersal events (RR: C->CG->C|G; C (52.87%), A (28.74%), G (9.04%), AC (6.03%), MO (3.32%); p*P* = 0.52; node 6). The Hydrothelphusini originated in Madagascar (RR: B->B^B->B|B; B (96.71%), MO (3.30%), p*P* = 0.96; node 7).

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The rest of the continental Potamonautidae had their ancestral range in East Africa (RR: C->C^C->C|C; C (94.50), MO (5.5%); p*P* = 0.94; node 8). With an ancestral range of East Africa (C), the East African *Potamonautes* and *Potamonautes calcaratus* (southern Africa) had a RR of C->CF->C|F (C = 99.39%, MO = 0.61%; p*P* = 0.99; node 9), with one vicariance and two dispersal events. There were no dispersal or vicariance events between *Platythelphusa* and *Potamonautes loveni* (RR: C->C^C->C|C; C (99.83), MO (0.17); p*P* = 1.00; node 10). One vicariance and two dispersal events were observed for the split of east African *Potamonautes kundondo* (Ethiopia) and *Potamonautes idjiwiensis* (DRC, Central Africa) (RR: C->DC->D|C; C (99.11%), MO (0.89%); p*P* = 0.99; node 11). The split between the East African (Kenya and Tanzania), West African (Mali), and Central African (undescribed DRC species) had the following RASP route: C->AC->A|C (C = 95.01%, MO = 0.55%; p*P* = 0.92; node 12), with one vicariance and two dispersal events. The ancestral range of the two undescribed DRC species and the East / West African *Potamonautes* species had a RASP route of C->CD->C|D (C = 96.39%, CD = 2.32%, D = 0.91%, MO = 0.39%; node 13). The predominantly southern African *Potamonautes* clade's ancestral range was in East Africa when this clade split from the East African *Potamonautes* species (RR: C->CF->C|F; C (92.97%), CF (6.12%), F (0.68%), MO (0.23%); p*P* = 0.88, node 14), with one vicariance and two dispersal events. The ancestral range of the South African *Potamonautes* was F->F^F->F|F (F = 94.31%, CF = 5.49%, MO = 0.2%; p*P* = 0.93, node 15), with no dispersal or vicariance events. The next dispersal (two events) / vicariance (1 event) occurred with the split of South and East African *Potamonautes* (RR: F->FC->F|C; F (98.23%), CF (1.59%), MO (0.18%); p*P* = 0.98; node 16). The RASP route of the ancestor of South African, and West African (Angola) / East African (Uganda) *Potamonautes* was F->AF->A|F (F = 96.16%, AF = 3.47%, A = 0.18%, MO = 0.19%; p*P* = 0.92; node 17), with one vicariance and two dispersal events. A West African ancestral range was inferred for the split between the Angolan (West Africa) freshwater crabs (*Potamonautes achietae* and three undescribed species) and the Ugandan (East Africa) *Potamonautes rukwanzi* and had one vicariance and two dispersal events (RR: A->CA->C|A; A (97.53%), AC (2.26%), C (0.14%), MO (0.07%); p*P* = 0.98, node 18). The last clade (node 19) had a South African ancestral range (RR: F->F^F->F|F; F (99.78%), MO (0.22%); p*P* = 1.00).

The phylogenetic reconstructions and BEAST tree topologies, to some extent, corroborated Linder *et al.*'s (2012) ecoregion phenogram (Fig. 5 A, B, C). I found that the basal clade (Fig. 5A), which comprised specimens from West Africa matched Linder *et al.*'s (2012) Congolian region (Fig. 5C (I). While, Linder *et al.* (2012) found the Congolian clade

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to be sister to the rest of the continental African taxa, I recovered a sister relationship between continental Africa and the Deckeniinae (which include three continental genera). Linder *et al*. (2012) grouped the Central and some of the West African taxa under the same clade (Fig. 5C (I)) and the ancestral reconstruction results support the phenogram, where the West African taxa feature within the different phylogenetic groups (blue sub-clades, Fig. 5A). Even with the exclusion of the Malagasy and Seychelles (Linder *et al*., 2012), I found sister relationship between the West and East African freshwater crab species (Fig. 5A, C (III, IV, V).

DISCUSSION

With the inclusion of 16 of the 18 Afrotropical freshwater crab genera, comprising 84 species, this study is to date the most comprehensive analysis of the phylogenetic relationships within the Potamonautidae and represents the most complete study on any freshwater crab biogeographical region. I found that the Potamonautidae were a monophyletic group with both ML bootstrap and BI posterior probability support, with a late Cretaceous divergence (107.57 – 96.04 Mya) and a West / East African ancestral range. Notably, following Cumberlidge *et al*.'s (2008) sub-family groupings, the Deckeniinae, comprising the Deckeniini (Seychelles / East Africa clade), Globonautina (*Afrithelphusa* / *Globonautes*) and the Hydrothelphusini (Malagasy freshwater crabs) were sister to the paraphyletic *Potamonautes* / *Platythelphusa* clade occurring on continental Africa, as opposed to the other continental freshwater crab taxa (*Liberonautes* / *Sudanonautes* / *Potamonemus*; Fig. 2, clade 1 and 2), which were the basal Potamonautinae. This relationship was also supported by the divergence dating analysis (Fig. 3). This raises more questions as to whether the Potamonautinae should in fact include the aforementioned West African genera.

Within the Deckeniinae, the addition of *Boreathelphusa*, which was not included in Daniels *et al*. (2006a), revealed its sister relationship to *Skelosophusa*. Moreover, *Hydrothelphusa* is not monophyletic, with *Marojejy longimerus* nested within the genus (clade 3b, Fig. 2). Unpublished data (Daniels, pers. comm.) suggests that the morphological taxonomy is inconsistent with genetically detected groups based on DNA data. Another instance of paraphyly was observed between *Potamonautes* (clades 4, 6, and 7) and *Platythelphusa* (clade 5, Fig. 2). The present study included eight *Platythelphusa* species, which confirmed the paraphyly of *Potamonautes* with *Platythelphusa*, as recovered by Daniels *et al*. (2006a) who only included one species of *Platythelphusa* (*P. armata*).

Platythelphusa is a monophyletic group within *Potamonautes*, but formed a sister relationship with *Potamonautes loveni* (clade 5, Fig. 2). Although *P. loveni* has been taxonomically problematic (Cumberlidge, 2010), there is no doubt that this species is morphologically divergent from *Platythelphusa* species. Because of its morphological divergence from other Afrotropical freshwater crabs, *Platythelphusa* was thought to have evolved from a separate invasion by a marine ancestor into Lake Tanganyika (African Rift Lake, East Africa), where it is endemic. However, there is no support for this assumption. Instead, *Platythelphusa* has complex origins, with no known extant relatives, and possibly evolved from a freshwater crab ancestor (Sternberg & Cumberlidge, 1999).

The same level of complexity has been observed among the endemic and morphologically diverse Lake Tanganyika's cichlid fishess (Koblmüller *et al*., 2008). Colonization of the lake by ancestral cichlids is deemed to have occurred roughly 12 – 20 Mya when some colonizing ancient fish species entered a proto-Lake Tanganyika (Salzburger *et al*., 2002), before present-day Lake Tanganyika, which is estimated to be 9 – 12 My old (Cohen *et al*., 1993). The cichlid fishes of Lake Tanganyika are believed to have radiated during the development of deep-water conditions in the Miocene approximately 9 – 12 Mya, becoming endemic to the lake and resulting in morphologically diverse tribes that are divergent from the rest of Africa's cichlids (Salzburger *et al*., 2002; Terai *et al*., 2003; Clabaut *et al*., 2005). The Miocene is also the period where many rifting, uplifts and drainage evolution were prevalent (Moore, 1999; Moore & Larkin, 2001; Stankiewicz & de Wit, 2006; Moore *et al*., 2009). These events, together with the age of Lake Tanganyika, are consistent with the divergence of *c*. 13.10 Mya (95% HPD: 5.81 – 20.70 Mya) for *Platythelphusa*, which also coincides with the diversification of cichlid fishes within the lake.

While phylogenetic affinities of the *Platythelphusa* cannot be accurately determined, I recovered two instances where there were sister relationships between geographically discrete East and West African freshwater crab species: within the Deckeniinae, i.e. the *Deckenia* (East Africa) / Globonautinae (West Africa) relationship (clade 3a, Fig. 2) and between *Potamonautes emini* from Tanzania (East Africa) and *P. ecorssei* from Mali (West Africa) was not reflected in the phylogenetic reconstructions, but emerged in the divergence estimations (West / East/ Central African clade, Fig. 3). For other taxa, close phylogenetic affinities between East and West African taxa are presumed to be a historical artefact of the continuous forest belt that stretched across Africa between the Oligocene and Miocene (approximately 30 – 25 Mya) (Clarke & Burgess, 2000). As such, it has been found that many taxa from the Eastern Arc Mountains (Kenya and Tanzania) are more closely related to

West and western-Central Africa taxa (i.e. the Guineo-Congolian rainforest) (Clausnitzer, 2003). Similarly, Lovett (1993) attributed the West African affinities of East African flora to the presumed historical connection of East African forests to the Guineo-Congolian lowland forests, and according to other studies (Roy *et al*., 1997; Burgess *et al*., 1998; Clarke *et al*., 2000) these affinities are relictual, as opposed to being a result of recent divergence. Here, I found that the East African *Deckenia* share close phylogenetic affinities with the west African genera (*Globonautes* and *Afrithelphusa*) from Guinea, with their West African ancestor having diverged through vicariance and dispersal (clade 5, Fig. 4) approximately 50.59 Mya (95% HPD: 44.95 – 98.18 Mya; Fig. 3). This Eocene date is much older than the Oligocene / Miocene suggested by Clarke & Burgess (2000). However, Africa's drainage systems underwent drastic rearrangements since the upper Cretaceous (until about 120 – 110 Mya) (Goudie, 2005; Stankiewicz & de Wit, 2006). Moreover, the end of the Cretaceous – early Tertiary was associated with high rainfall as a result of landmasses being surrounded by the warm waters of the Tethys Sea (Parrish *et al*., 1982). Maley (1996) ascribed these wet conditions to being the reason that West and western Central Africa were occupied by wet tropical forests during the Cretaceous and early Tertiary (Maley, 1996). Therefore, the conditions would have been ideal for the invasion of continental areas by a marine ancestor of freshwater crabs, which is presumed extinct (Sternberg *et al*., 1999).

The divergence time dating for the Potamonautidae was found to be in the Cretaceous where an unknown, possibly extinct marine, brachyuran ancestor settled in West Africa. This would support Bott's (1972) hypothesis that a marine crab ancestor living in the Tethys Sea may have entered Africa during the Tertiary, making a West African ancestry for the Potamonautidea plausible. And with Africa's anti-clockwise rotation (to its present-day position) and subsequent retreat and closure of the Tethys oceanic basins in the late Eocene (Goudie, 2005), it is possible that the marine ancestor remained trapped and progressively adapted to freshwater conditions. Various rifting and uplifting episodes were also prevalent during the Tertiary, in the Oligocene and especially during the Miocene (John, 1986), which is the period where most diversifications within the Potamonautidae were found to have occurred (Fig. 3). Similar findings have been reported for Afrotropical freshwater fish, where the speciation of the genus *Synodontis* during the Tertiary was attributed to African drainage evolution and the formation of the East African rift system (Pinton *et al*., 2013). Moreover, the split of the Deckeniinae from the Potamonautinae (*c*. 62.60 Mya, 95% HPD 59.21 – 104.60 Mya) coincides with that of cichlid divergence (41 – 56 Mya; Vences *et al*., 2001).

During the Tertiary, drainage changes occurred as a result of the flexuring of the Okavango (southern Africa) / Kalahari / Zimbabwe (eastern-southern Africa) axis, which led to the separation of other inland drainages basins (Moore, 1999; Moore & Larkin, 2001; Stankiewicz & de Wit, 2006; Moore *et al*., 2009). According to Stankiewicz & de Wit's (2006) model, this flexuring transformed the Okavango / Cuando (West Africa) / Upper Zambezi (south-central Africa) into a landlocked system. At the same time as the transformation of the Okavango-Cuando-Upper Zambezi, the watershed that was between the Congo basin and the rivers that drained into the Atlantic Ocean shifted eastwards (Moore, 1999). By the Pliocene, many rifting and uplift episodes occurred in East Africa and various other drainage systems were transformed or became landlocked (Stankiewicz & de Wit, 2006). The Congo basin remained landlocked throughout the early Miocene until the watershed to the Atlantic Ocean was breached in the Pleistocene (*c*. 1 Mya), resulting in contemporary drainage patterns (Stankiewicz & de Wit, 2006). I would therefore expect that freshwater crab fauna occurring in the Congo River drainage system will be unique, with high levels of endemism.

According to Cumberlidge (1999), the distribution patterns of freshwater crabs in West and Central Africa were (at least in part) influenced by Pleistocene climate changes that resulted in the contraction of widespread forests to small forest refugia in Upper Guinea (West Africa), Lower Guinea (Nigeria and Cameroon), and eastern Democratic Republic of the Congo. However, the results suggest an older (than Pleistocene) divergence for West and Central African freshwater crabs, between the late Cretaceous (95.60 Mya) for *Liberonautes* / *Sudanonautes* / *Potamonemus*, late Oligocene (26.22 Mya) for *Afrithelphusa* / *Globonautes*, up until the Miocene (20.55 Mya) for undescribed Angolan *Potamonautes* species and *P. achietae* (Fig. 3). According to Cumberlidge's (1999) hypothesis, the Congo River Basin, in Central Africa, is a possible centre of endemism and diversity for *Potamonautes*, and species diversity is said to decrease with increasing distance from this centre. Ironically, it is in this area where much of the taxonomic assignments need attention. Nevertheless, the validity of the putative freshwater crab regional ecotypes is far from established, and character polarity is still not clear enough to decide whether the predominantly aquatic lake and riverine taxa of western Central Africa are more morphologically plesiomorphic than the stream-living and semi-terrestrial species (Cumberlidge, 1999). Moreover, while the Congo basin is said to be a centre of freshwater crab diversity (Cumberlidge, 1999), this study is taxon poor for this region. For this reason, the centre of origin hypothesis, which states that freshwater crab fauna originated from Central Africa, cannot be refuted because of the large (Central African)

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sampling gap between East and West Africa. Therefore, future sampling endeavours should focus on the freshwater crab fauna of the Congo basin.

At present, I do not have concrete evidence (as a result of poor sample sizes from Central Africa) to fully accept or reject the plausibility of a West African ancestor because the ancestral reconstructions suggested a higher possibility of an East African ancestor followed by West Africa. However, I tend to lean towards a West African origin because the phylogenetic reconstructions (Fig. 2) and divergence dating (Fig. 3) suggested that the West African taxa (*Liberonautes*, *Sudanonautes*, and *Potamonemus*) were the most basal / ancestral group. However, as with the Central African freshwater crab fauna, *Sudanonautes* is taxon poor in this study and I believe that *Potamonemus* also has undiscovered diversity. I deduce that the dramatic (climatological and drainage system) transformations experienced by continental Africa (Maley, 1996) towards the end of the Tertiary (Moore, 1999; Moore & Larkin, 2001; Stankiewicz & de Wit, 2006; Moore *et al*., 2009) leading to rainforest contraction (Burgess & Clarke, 2000) that, together with drainage evolution through the isolation of the Congo basin, resulted in the split of the West African freshwater crab taxa from those that may have already migrated eastward. These would be the relicts of global change and ultimately giving rise to *Potamonautes* (51.61 Mya; 95% HPD: 39.07 – 75.06 Mya), through vicariance and dispersal from as an East African ancestor (clade 9, Fig. 4). In due course, the East African freshwater crab taxa became species rich as a result of the rifting episodes that were experienced by the Eastern Arc Mountains.

Indeed, the majority of the currently described *Potamonautes* species are from East Africa (Table 2) and speciation reflects the intricate geological history of the east African rift valleys. *Potamonautes* comprises several clades and requires taxonomic revision. A molecular phylogeny including all of the ±122 currently known *Potamonautes* species would allow for the revisitation of the morphology in order to morphologically distinguish species occurring in the different clades. However, the revision of *Potamonautes* is outside the scope of this research because it would require that all species within this genus be included in the analysis, which I currently do not have.

Geographically, East Africa is proximal to the Indian Ocean islands of Madagascar and the Seychelles; however, I found West African affinities for the Deckeniinae (clade 4, Fig. 4). The occurrence of these relationships post-dates the breakup of Gondwana, whereby both Madagascar and the Seychelles were completely isolated from the African continent by 80 – 60 Mya (Rabinowitz *et al*., 1983; Reeves *et al*., 2002; Yoder & Nowak, 2006). The occurrence of taxa that are closely related to African freshwater crab taxa may have resulted

from accidental transoceanic oceanic dispersal (Cumberlidge, 2008) through rafting of the ancestral relict from West Africa. Close phylogenetic affinities between continental African and Malagasy or Seychelles taxa have been reported, for example, amphibians (Vences *et al*., 2004, Vences *et al*., 2003), reptiles (Raxworthy *et al*., 2002), plants (including bryophytes and angiosperms; Pocs, 1998; Burgess *et al.*, 1998; Couvreur *et al*., 2008; Ali *et al*., 2013), as well as mammals (Yoder *et al*., 2003). Daniels *et al*. (2006a) and Cumberlidge *et al*. (2008) suggested that transoceanic rafting may be conceivable considering that some contemporary freshwater crab species have been shown to tolerate a limited degree of exposure to saline environments (Morris & van Aard, 1998), up to 50% concentration of seawater (Shaw, 1959). Bearing in mind that the ancestral origins of the Potamonautidae are possibly from an extinct marine ancestor, it is credible to hypothesize that the Seychelles and Malagasy freshwater crab ancestors were more tolerant to highly saline habitats than their modern-day counterparts (Cumberlidge, 1999; Daniels *et al*., 2006a), which could also be a reason why there are no freshwater crab fauna in recently formed (less than ± 10 My old) islands that are adjacent to continental Africa (Cumberlidge, 2008).

A late Cretaceous divergence of freshwater crab fauna post-dates the split of Gondwana as previously reported by other studies (Daniels *et al*., 2006a; Klaus *et al*., 2011). Daniels *et al*. (2006a) refuted a Gondwanan origin for the Potamonautidae, while Klaus *et al*. (2011) found that the Asian-Australian Gecarcinucidae also did not conform to Gondwanan origins. Furthermore, chichlid fishes, which are perceived as model freshwater taxa for speciation and evolutionary histories (Kocher, 2004; Seehausen, 2006), were also found to have diverged post-Gondwanan break up (between 65 and 57 Mya) (Friedman *et al*., 2013). The divergence dates are slightly older than Daniels *et al*.'s (2006a) 78.6 – 75.03 Mya (also in the late Cretaceous). This may be due to the different calibration methods used. Daniels *et al*. (2006a) used various calibration points based on fossils and the geological age of the Seychelles. In this study, I used the mean mutation rates of the genetic markers because there are no reliable fossil records for the Potamonautidae. The fossil from Kenya is dated to the Miocene, which post-dates the divergence the Potamonautidae. Nevertheless, divergence time estimation dates should be interpreted with caution because high 95% HPD ranges around the divergence dates reflect the high level of uncertainty of this method.

The complexity of the phylogeny of the Potamonautidae is far from resolved, but the addition of more representative genera and species per genus provided a better resolution to the phylogenetic relationships within this family. It is however evident that the diversification of the Potamonautidae is consistent with the hydrological, vegetation structure (historical

forests), geological and climatic events that occurred from the Upper Cretaceous onwards, which were instrumental in shaping the speciation of the Potamonautidae. A lack of samples from the so named centre of endemism (Central Africa) remains a setback in the resolution of the Potamonautidae's sub-family level taxonomy, especially regarding relationships within the Patamonautinae, *Liberonautes* / *Sudanonautes* / *Potamonemus* and *Potamonautes* and the nesting of the Deckeniinae between the former three genera and the latter one. The inclusion of *Erimetopus*, which is sister to the former three West African genera (Daniels, pers. comm.), may illuminate these relationships further. In addition, at present, I am not certain of the phylogenetic placement of *Erimetopus* because of a lack of useable genetic material. It will therefore be beneficial to conduct a sampling survey to search for fresh and usable specimens. For the present study I could not obtain more specimens from Central Africa because the region is precarious, with constant and ongoing political instability and social unrest, which makes it logistically difficult and unsafe for sampling. However, specimens from this drainage system are currently being collected (Daniels, pers. comm.), which should provide some resolution to the phylogenetic relationships and ancestral affinities of the Potamonautidae.

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Table 1 The sub-families within Potamonautidae and the placement of extant genera within the sub-families. Genus names in bold refer to incorrect placements by (De Grave *et al.*, 2009).

Sub-family	Placement of genera (Cumberlidge *et al.*, 2008)	Placement of genera (De Grave *et al.*, 2009)
Potamonautinae Bott, 1970a	*Erimetopus* Rathbun, 1894	*Erimetopus*
		Foza
	Liberonautes Bott, 1955	*Liberonautes*
	***Louisea* Cumberlidge, 1994**	
	Potamonautes Bott, 1970a	*Potamonautes*
	Potamonemus Cumberlidge & Clark, 1992	*Potamonemus*
	Sudanonautes Bott, 1955	*Sudanonautes*
	Platythelphusa A. Milne-Edwards, 1887	*Platythelphusa*
Deckeniinae Ortmann, 1897		
Deckeniini Ortmann, 1897	*Deckenia* Hilgendorf, 1869	*Deckenia*
	Seychellum Ng, Števčić & Pretzmann, 1995	*Seychellum*
Globonautina Bott, 1969	*Globonautes* Bott, 1959	
	Afrithelphusa Bott, 1969	
Hydrothelphusini Bott, 1955	*Boreathelphusa* (*Boreas*) Cumberlidge & Sternberg, 2002	
	***Foza* Reed & Cumberlidge, 2006**	
	Hydrothelphusa A. Milne-Edwards, 1872	
	Madagapotamon Bott, 1965	
	Malagasya Cumberlidge & Sternberg, 2002	
	Marojejy Cumberlidge, Boyko & Harvey, 2000	
	Skelosophusa Ng & Takeda, 1994	
Hydrothelphusinae Bott, 1955		*Afrithelphusa*
		Boreathelphusa
		Globonautes
		Hydrothelphusa
		Louisea
		Madagapotamon
		Malagasya
		Marojejy
		Skelosophusa

Table 2 Specimens included in this study, including the country from which they were collected, museum accession numbers refers to the location of the holotypes: NMU = Northern Michigan University, FMNH, Field Museum of Natural History (Chicago), SAM = Iziko South African Museums (Cape Town), and the GenBank accession numbers (where available) for each genetic marker (mtDNA: 12S rRNA, 16S rRNA, COI; and nuDNA: H3). * = Democratic Republic of Congo.

Species	**Country**	**Museum Accession number**	**GenBank accession numbers**				**Reference**
			12S Rrna	16S rRNA	COI	Histone 3	
Afrithelphusa monodosa	Guinea	NMU 25.IV.2005.C	This study	This study	This study	This study	This study
Boreathelphusa uglowi	Madagascar	FMNH 5732	This study	This study	This study	This study	This study
Deckenia imitatrix	Kenya	NMU 1998.1	AY 803503	AY 803544	AY 803576	AY 803698	Daniels *et al*., 2006a
Deckenia mitis	Tanzania	-	This study	This study	This study	This study	This study
Foza raimundi	Madagascar	FMNH 7438	This study	This study	This study	This study	This study
Globonautes macropus	Guinea	NMU VII. 1988	This study	This study	-	-	This study
Hydrothelphusa agilis	Madagascar	FMNH 5729	AY 803505	AY 803546	AY 803578	AY 803700	Daniels *et al*., 2006a
Hydrothelphusa bombotokensis	Madagascar	FMNH 6878	AY 803506	AY 803546	-	AY 803701	Daniels *et al*., 2006a
Hydrothelphusa venci	Madagascar	FMNH 13940	This study	This study	This study	-	This study
Hydrothephusa goudoti	Madagascar	FMNH 4652	AY 803507	AY 803548	AY 803579	AY 803702	Daniels *et al*., 2006a
Hydrothephusa madagascariensis	Madagascar	FMNH 7438	AY 803508	AY 803549	AY 803580	AY 803703	Daniels *et al*., 2006a
Johara tiomanensis	Malaysia	ZRC 1999.0899	AY 803517	AY 803556	This study	AY 803712	Daniels *et al*., 2006a
Liberonautes chaperi	Liberia	NMU VII.1988a.1	This study	This study	-	This study	This study
Liberonautes latidactylus	Liberia	-	This study	This study	This study	This study	This study
Liberonautes lugbe	Guinea	Unaccessioned	This study	This study	This study	This study	This study
Liberonautes nanoides	Liberia	NMU 14.XII.1988	This study	This study	-	This study	This study
Liberonautes nimba	Liberia	SAM A 48202	This study	This study	This study	This study	This study
Liberonautes rubrigimanus	Liberia	SAM A 48201	This study	This study	This study	This study	This study
Madagapotamon humberti	Madagascar	FMNH 11049	AY 803509	AY 803550	-	AY 803704	Daniels *et al*., 2006a
Malagasya antongolensis	Madagascar	-	AY 803511	AY 803551	-	AY 803706	Daniels *et al*., 2006a
Malagasya goodmani	Madagascar	-	AY 803512	-	This study	AY 803707	Daniels *et al*., 2006a

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Table 2 continued

Marojejy longimerus	Madagascar	FMNH 4656	AY 803513	AY 803552	AY 803582	AY 803708	Daniels *et al*., 2006a
Platythelphusa armata	Tanzania	ZMA Crust.De.204685	AY 803491	AY 803531	This study	AY 803607	Daniels *et al*., 2006a
Platythelphusa conculcata	Tanzania	Unaccessioned	DQ 203190	DQ 203218	-	-	Marijnissen *et al*., 2006
Platythelphusa denticulata	Tanzania	Unaccessioned	DQ 203194	DQ 203220	-	-	Marijnissen *et al*., 2006
Platythelphusa echinata	Tanzania	Unaccessioned	DQ 203196	DQ 203222	-	-	Marijnissen *et al*., 2006
Platythelphusa immaculata	Tanzania	Unaccessioned	DQ 203198	DQ 203224	-	-	Marijnissen *et al*., 2006
Platythelphusa maculata	Tanzania	Unaccessioned	DQ 203203	DQ 203228	-	-	Marijnissen *et al*., 2006
Platythelphusa praelongata	Tanzania	Unaccessioned	DQ 203206	DQ 203232	-	-	Marijnissen *et al*., 2006
Platythelphusa tuberculata	Tanzania	Unaccessioned	DQ 203204	DQ 203230	-	-	Marijnissen *et al*., 2006
Potamon fluviatilis	Italy	Unaccessioned	AY 803515	AY 803554	AY 803584	AY 803710	Daniels *et al*., 2006a
Potamonautes achietae	Angola	-					
Potamonautes barbarai	South Africa	SAM A41012	This study	This study	This study	This study	Phiri & Daniels, 2014
Potamonautes barnardi	South Africa	SAM A41060	This study	This study	This study	This study	Phiri & Daniels, 2014
Potamonautes bayonianus	Botswana	-	AY 042321	AY 042243	AF 510868	This study	Daniels *et al*., 2002
Potamonautes bellarussus	Mozambique	SAM A48218	This study	This study	This study	This study	This study
Potamonautes brincki	South Africa	-	AY 042322	AY 042244	AF 510875	AY 803674	Daniels *et al*., 2002
Potamonautes calcaratus	South Africa	-	AY 042323	AY 042242	AF 510867	AY 803675	Daniels *et al*., 2002
Potamonautes choloensis	Mozambique	SAM A46802	JF 799164	JF 799214	JF 799210	This study	Daniels & Bayliss, 2012
Potamonautes clarus	South Africa	-	AY 042320	AY 042241	AF 510872	AY 803676	Daniels *et al*., 2002
Potamonautes dentatus	South Africa	-	AY 042324	AY 042246	AF 510878	AY 803677	Daniels *et al*., 2002
Potamonautes depressus	South Africa	-	AY 042325	AY 042247	AF 510877	AY 803678	Daniels *et al*., 2002
Potamonautes ecorssei	Mali	NMU 07.01.2003.1	AY 803492	AY 803532	-	AY 803679	Daniels *et al* 2006a
Potamonautes emini	Tanzania	ZMA Crust. De. 204680	AY 803493	AY 803533	-	AY 803680	Daniels *et al*., 2006a
Potamonautes flavusjo	South Africa	SAM A48207	This study	This study	This study	This study	This study
Potamonautes gerdalensis	Zambia	-	This study	This study	This study	This study	This study
Potamonautes granularis	South Africa	-	AY 042326	AY 042254	AF 510876	AY 803681	Daniels *et al*., 2002
Potamonautes idjiwiensis	DRC*	SAM A	This study	This study	This study	This study	This study
Potamonautes kundudo	Ethiopia	SAM A 48200	This study	This study	This study	This study	This study

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Table 2 continued

Potamonautes lirrangensis	Malawi	ZMA Crust. De. 204681	AY 803494	AY 803534	AY 803568	AY 803682	Daniels *et al*., 2006a
Potamonautes lividus	South Africa	-	AY 042327	AY 042248	AF 510879	AY 803683	Daniels *et al*., 2002
Potamonautes loveni	Kenya	BMNH 2004.1-6	This study	This study	This study	This study	This study
Potamonautes montivagus	Mozambique	-	This study	This study	This study	This study	This study
Potamonautes mulanjeensis	Malawi	SAM A46801	JF 799175	JF 799139	JF 799201	-	Daniels & Bayliss 2012
Potamonautes mutareensis	Zimbabwe	SAM A45934	KC768246	KC768273	KC768299	This study	Phiri & Daniels 2014
Potamonautes namuliensis	Mozambique	SAM A46797	JF 799170	JF 799140	JF 799196	This study	Daniels & Bayliss 2012
Potamonautes niloticus	Tanzania	ZMA Crust. De. 204683	AY 803469	AY 803536	This study	AY 803685	Daniels *et al*., 2006a
Potamonautes obesus	Tanzania	-	AY 803497	AY 803537	AY 803647	AY 803686	Daniels *et al*., 2006a
Potamonautes odheneri	Tanzania	NMU 14.07.2004	AY 803498	AY 803538	AY 803571	AY 803687	Daniels *et al*., 2006a
Potamonautes parvicorpus	South Africa	-	AY 042328	AY 042252	AF 510869	AY 803688	Daniels *et al*., 2002
Potamonautes parvispina	South Africa	-	AY 042329	AY 042253	AF 510873	AY 803689	Daniels *et al*., 2002
Potamonautes perlatus	South Africa	-	AY 042330	AY 042249	AF 510874	AY 803690	Daniels *et al*., 2002
Potamonautes platynotus	Tanzania	NMU 23.04.2003.6	AY 803499	AY 803539	AY 803572	AY 803691	Daniels *et al*., 2006a
Potamonautes raybouldi	Tanzania	ZMA Crust. De. 204684	AY 803540	AY 803500	AY 803573	AY 803692	Daniels *et al*., 2006a
Potamonautes rukwanzi	Uganda	NMU 16.VII.1993b.1	This study	This study	-	This study	This study
Potamonautes sidneyi	South Africa	-	AY 042331	AY 042245	AF 510871	AY 803693	Daniels *et al*., 2002
Potamonautes sp. 1 (ES07-F326)	Angola	-	This study	This study	This study	-	This study
Potamonautes sp. 2 (ES07-F451)	Angola	-	This study	This study	This study	This study	This study
Potamonautes sp. 3 (ES08-B007)	Angola	-	This study	This study	This study	-	This study
Potamonautes sp. 4 (West Mt. Kenya)	Kenya	-	This study	This study	This study	This study	This study
Potamonautes sp. 5 (UGSB 11408)	DRC	-	This study	This study	-	This study	This study
Potamonautes sp. 6 (UGSB 5563)	DRC	-	This study	This study	This study	-	This study
Potamonautes subukia	Kenya	NMU 18.10.2003	AY 803495	AY 803535	AY 803569	AY 803684	Daniels *et al*., 2006a
Potamonautes suprasulcatus	Tanzania	BMNH not registered	This study	This study	This study	This study	This study
Potamonautes unispinus	South Africa	-	AY 042332	AY 042250	AF 510870	AY 803694	Daniels *et al*., 2002
Potamonautes warreni	South Africa	-	AY 042333	AY 042251	AF 510880	AY 803695	Daniels *et al*., 2002
Potamonemus asylos	Cameroon	BMNH 1994.588-591	This study	This study	This study	This study	This study

Table 2 continued

Potamonemus mambilorum	Cameroon	BMNH 1991.183	This study	This study	-	-	This study
Potamonemus sachsi	Cameroon	NMU 09.04.1983	AY 803490	AY 803530	-	-	Daniels *et al*., 2006a
Seychellum silhouette	Seychelles	SAM A48220	This study	JF 799294	JF 799368	This study	Daniels 2011, this study
Seychellum mahefregate	Seychelles	SAM A48226	This study	JF 799277	JF 799351	This study	Daniels 2011, this study
Seychellum alluaudai	Seychelles	SAM A48235	This study	JF 799260	JF 799319	This study	Daniels 2011, this study
Skelosphusa eumeces	Madagascar	FMNH 11059	AY 803514	AY 803553	AY 803583	AY 803709	Daniels *et al*., 2006a
Skelosphusa prolixa	Madagascar	FMNH 7596	This study	This study	This study	This study	This study
Socotra socotrensis	Socotra	NMU 10.1998.1	AY 803516	AY 803555	AY 803585	AY 803711	Daniels *et al*., 2006a
Sudanonautes aubryi	Nigeria	NMU 23.04.1984A	AY 803502	AY 803542	AY 803575	-	Daniels *et al*., 2006a
Sudanonautes floweri	Gabon	-	AY 803501	AY 803541	AY 803574	AY 803696	Daniels *et al*., 2006a

Table 3 Substitution model parameters (jModelTest v. 2.1.3) for each locus used in the Bayesian inference (BI) and divergence time estimations analyses.

Gene fragment	Fragment length (base pairs)	Model	Base pair frequencies (%)	Gamma (G) distribution parameter	Proportion invariable (I) sites
12S rRNA	366	TPM2uf + I + G (nst = 6; -lnL = 6698.56; AIC = 13755.12)	A = 43.73 C = 4.90 G = 13.55 T = 37.82	0.678	0.155
16S rRNA	446	GTR + I+ G (nst = 6; -lnL = 10553.93; AIC = 21471.86)	A = 37.32 C = 9.50 G = 16.39 T = 36.79	0.585	0.202
			T = 35.4		
COI	600	TVM + I + G (nst = 6; -lnL = 10705.91; AIC = 21773.81)	A = 41.41 C = 4.98 G = 17.22 T = 36.40	0.276	0.280
H3	300	TPM2uf + G (nst = 6; -lnL = 2784.089; AIC = 5924.18)	A = 19.83 C = 26.63 G = 30.03 T = 23.51	0.220	N/A

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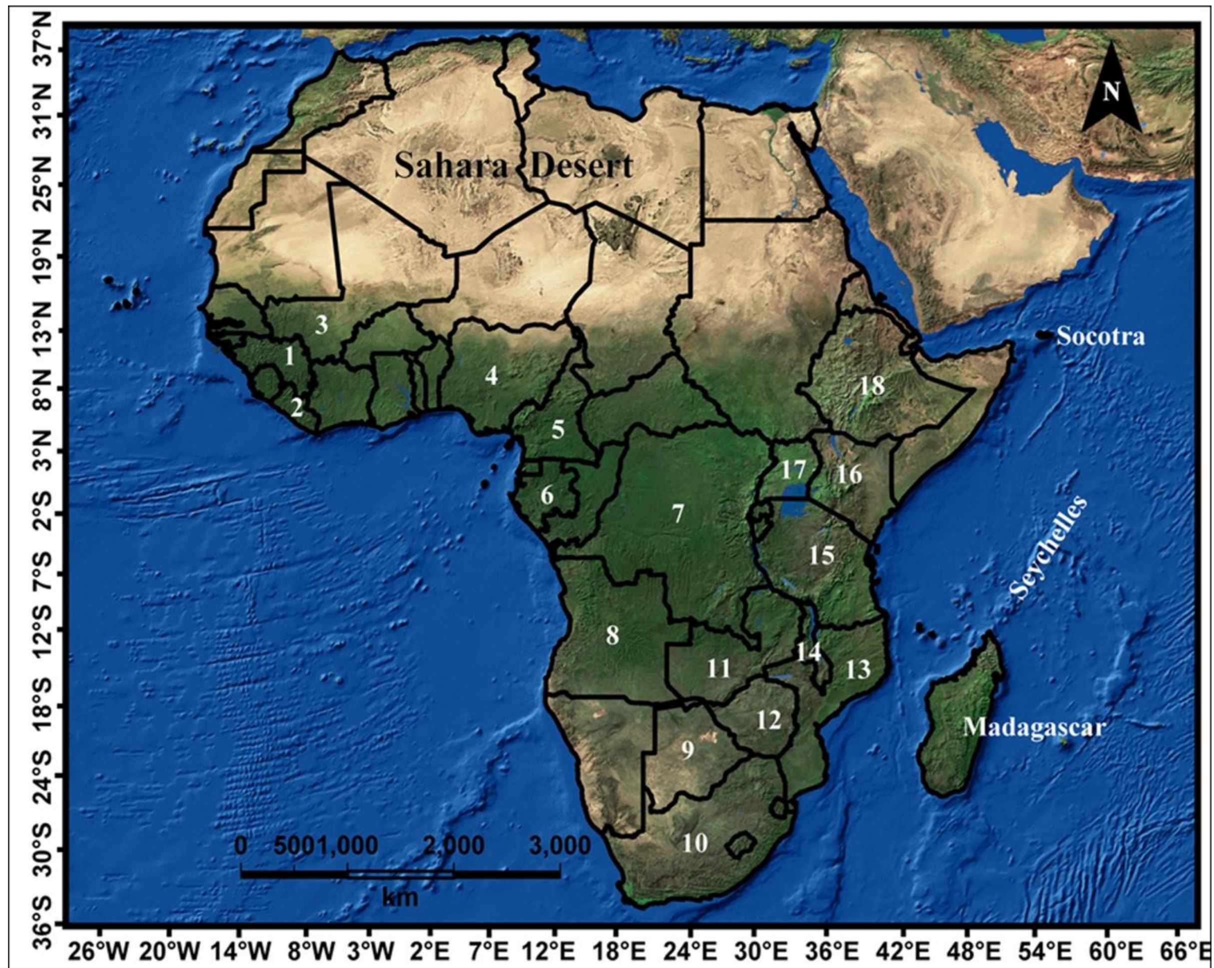

Figure 1 The Afrotropical region (continental Africa, Madagascar, Seychelles, and Socotra) with countries from where specimens were available for this study: 1 = Guinea, 2 = Liberia, 3 = Mali, 4 = Nigeria, 5 = Cameroon, 6 = Gabon, 7 = Democratic Republic of Congo (DRC), 8 = Angola, 9 = Botswana, 10 = South Africa, 11 = Zambia, 12 = Zimbabwe, 13 = Mozambique, 14 = Malawi, 15 = Tanzania, 16 = Kenya, 17 = Uganda, and 18 = Ethiopia.

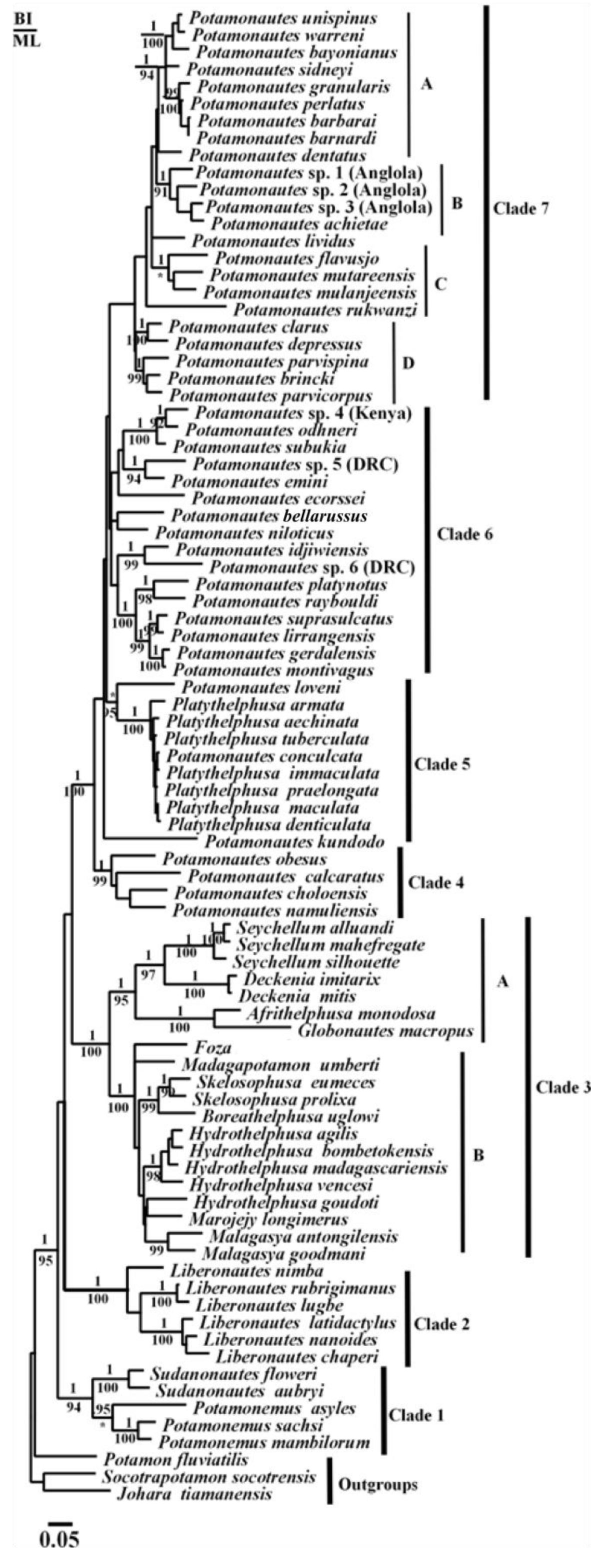

Figure 2 A maximum likelihood (ML) representation of the phylogenetic reconstruction of the Afrotropical Potamonautidae constructed from the combined mt- and nuDNA (12S rRNA, 16S rRNA, COI, and H3) dataset. Node support is indicated by BI posterior probability values 0.95 (above the branches) and ML bootstrap values ≥75% (below the branches), and * indicates no support.

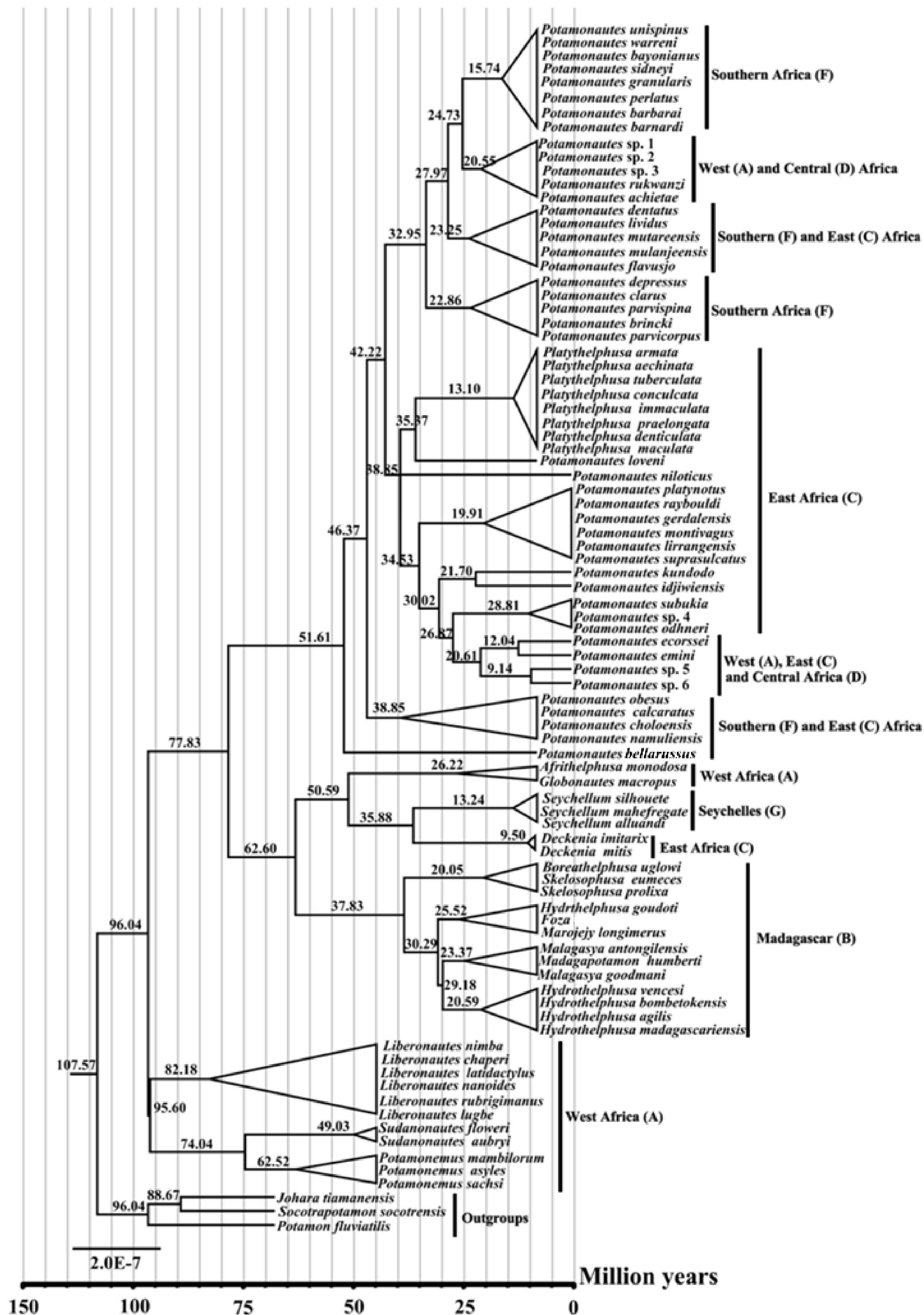

Figure 3 Divergence time estimations for the Afrotropical Potamonautidae inferred from the combined mt- and nuDNA (12S rRNA, 16S rRNA, COI, and H3) dataset. Numbers above branches represent divergence time in million years (95% highest posterior density (HPD) values are in text for the divergence dates that are discussed in the Results section).

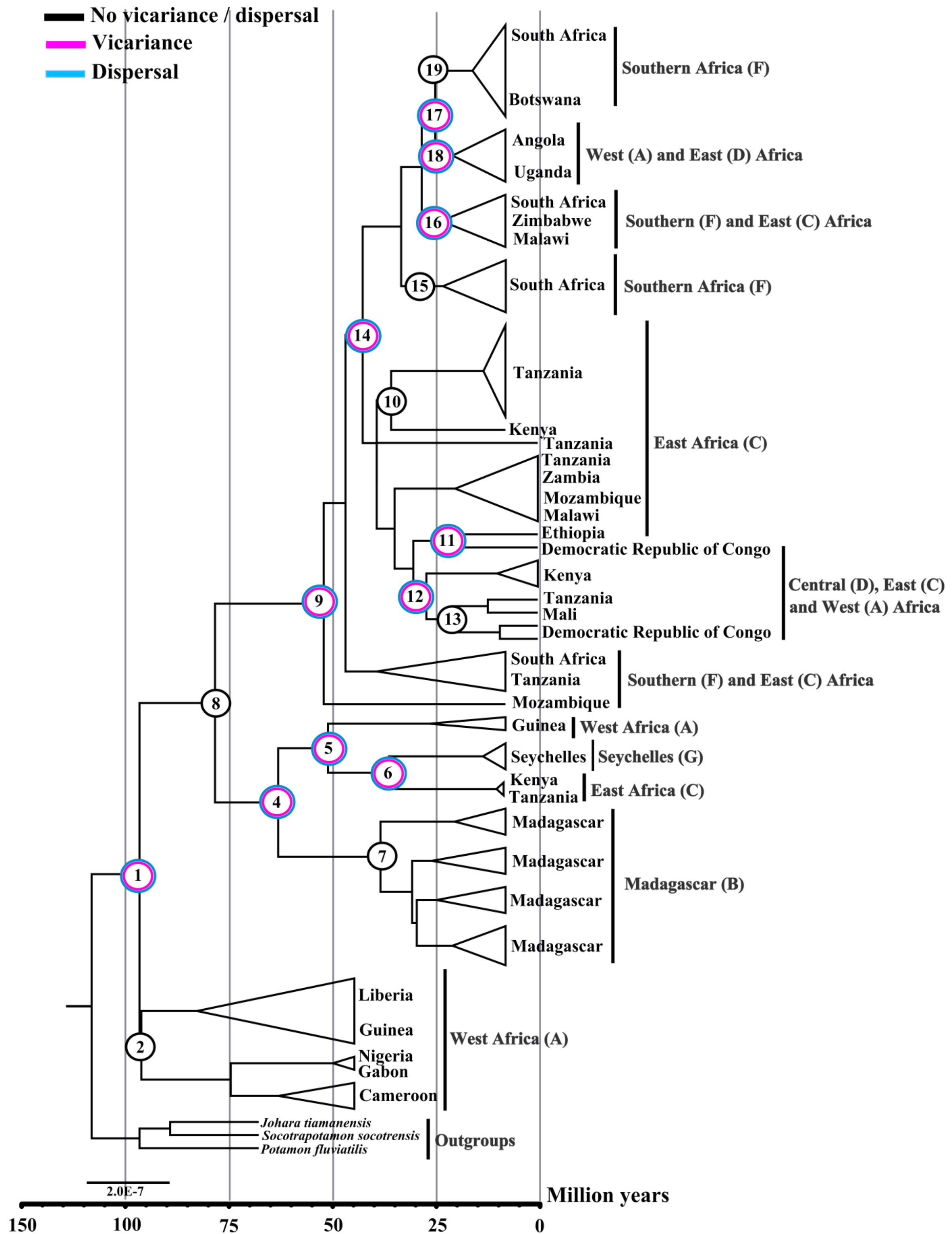

Figure 4 Ancestral area reconstruction for the Potamonautidae using Bayesian Binary Method in RASP. The node numbers represent the key nodes that are referred to in the Results section.

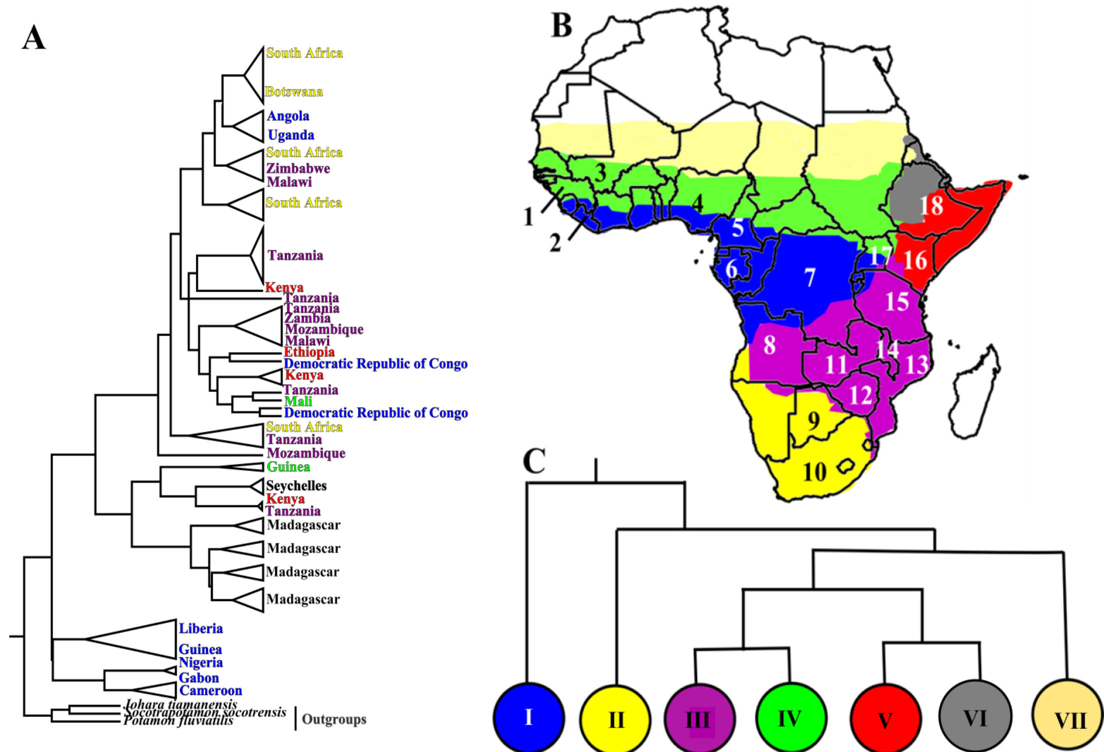

Figure 5 A comparison of the phylogenetic relationships of the Potamonautidae (A) (using the BEAST topology output) to Linder's (2012) ecoregions (B) and proposed phenogram (C). B and C are modified from Linder *et al*. (2012). In the phenogram (C), I = Congolian region (Congo and Guinea), II = Southern Africa (KwaZulu-Natal, Kalahari, Cape) and South-West Angola regions, III = Zambezian region, IV = Sudanian region, V = Somalian Region, VI = Ethiopian region, and VII = Saharan region (not included in the present study because freshwater crabs are not known to occur in this region).

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CHAPTER 7

SYNTHESIS

In this research, the phylogeny of the Afrotropical (sub-Saharan Africa, Madagascar, and Seychelles) freshwater crab family Potamonautidae was revisited in order to obtain a better resolution of the current standings of the phylogenetic relationships within the family and to infer biogeographical histories. The inclusion of 84 species from 16 out of 18 genera makes this the most comprehensive analysis of the phylogeny and biogeography of freshwater crab fauna in the Afrotropical region. Multiple genetic markers: mitochondrial (12S rRNA, 16S rRNA, COI) and nuclear (28S rRNA, DecapANT, histone 3, PEPCK) DNA were used. Moreover, the evolutionary histories of the Potamonautidae was inferred by also reconstructing their ancestral ranges and estimating their divergence time at various spatial scales, i.e. regional (Western Cape and KwaZulu-Natal provinces of South Africa) and continental (Afrotropical region).

The main findings were that the Potamonautidae originated in West or East Africa in the late Cretaceous. This means that the Gondwanan origins of the Afrotropical freshwater crab fauna can be refuted as previously pointed out by Daniels *et al* (2006a). However, while the ancestral reconstructions analysis of the Potamonautidae suggested a higher probability of a freshwater ancestor to have entered the African continent in the east, I believe that this finding should, at least for now, be carefully interpreted because of the poor taxon sampling from Central Africa. Obtaining more specimens from Central Africa, a supposed centre of diversity, may further elucidate ancestral origins of the family. I hypothesize that the true ancestral range is more likely to be West Africa as the divergence time estimation and phylogenetic reconstructions suggest, and the inclusion of *Louisea* and more *Sudanonautes* species is likely to corroborate this idea. In addition, perhaps more specimens from Central African this will also shed some light on the East / West phylogenetic relationships (i.e. *Deckenia* and Globonautinae). Moreover, *Erimetopus* has never been subjected to molecular phylogenetic studies, and it would therefore be interesting to ascertain its true phylogenetic identity once a usable specimen is obtained.

The exclusion of *Louisea*, *Erimetopus*, and some Central African specimens was not the only shortfall. I only managed to use 39 out of the approximately 122 described *Potamonautes* species. This genus is the most widespread on the continent and although it is its biogeographical affinities lie in East Africa, some assignments at the species level have been found to be artificial, with the prevalence of cryptic lineages within presumably

widespread species (Chapter 2 and Chapter 3). Because I only used one specimen per species per collection locality as provided to me by museum curators, there is no way of knowing how prevalent the level of cryptic speciation is within this genus. An example being *Potamonautes perlatus* s.s., which was historically considered to have a wide distribution range, spanning across West, Central, and southern Africa. But as shown in Chapter 2, *P. perlatus* s.s. only occurs in western flowing drainages of the Cape Fold Mountain range (Western Cape Province, South Africa). Moreover, the advancement of analytical methods and tools, compared to past studies, revealed even more widespread cryptic speciation within two *Potamonautes* species (*P. clarus* and *P. depressus*), with some conservation implications. Six novel lineages were discovered from the morphologically ambiguous *P. clarus* / *P. depressus* species complex within a small geographic area. Considering that very few fine-scale sampling efforts have been carried out on continental Africa, more freshwater crab diversity is yet to be recovered. However, extensive geographic sampling is required in order to realize this objective.

Conversely, there are instances where the genus can show high levels of endemism, with some species restricted to narrow geographic areas. For example, *P. mutareensis* from the East African Highlands of Zimbabwe was found to occur in five rivers and did not occur in sympatry with *P. unispinus* (Chapter 4) even though their distribution ranges overlapped. Also, the two novel species described in Chapter 5 are considered to be endemic to Niassa Province in Mozambique (*P. bellarussus*) and Mpumalanga Province in South Africa (*P. flavusjo*). In total, this study, spanning three years has yielded 11 novel *Potamonautes* species (i.e. *P. barbarai*, *P. barnardi*, *P. bellarussus*, *P. flavusjo*, *P. mutareensis*, and six *Potamonautes* sp. nov. from the Drakensberg Mountain Range (Chapter 3) that I will describe in the near future.

Further research:

This research has major potential for expansion, but expansion is currently beyond the scope of this thesis given the three year timeframe. The following objectives should be focal points in future research:

- Focusing on obtaining more specimens from Central Africa, which is already underway. There are also prospects to obtain more specimens from West Africa (specifically from Cameroon) and East / Central Africa (Uganda).

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- Devising reliable methods to morphologically delineate the six novel cryptic lineages recovered in Chapter 3. In Chapter 2, I showed that gonopods, which are currently the most widely used morphological characters, are not useful at delimiting closely related or recently diverged lineages.
- Exploring cryptic differentiation in widely distributed *Potamonautes* species (e.g. *P. niloticus* and *P. bayonianus*)
- Conducting a meta-analytical study that includes other freshwater taxa to discern whether freshwater crabs can be used as evolutionary models or for delineating ecoregions.
- Conducting cladistic analyses of morphological characters within the Potamonautidae to delineate the generic boundaries of *Potamonautes.*
- Undertaking focal sampling of high mountain areas to document diversity.
- Employing phylogenetic diversity in order to develop conservation management plans for the Potamonautidae.
- Including freshwater crabs together with other freshwater invertebrate taxa, such as molluscs and Odonata, to identify areas of conservation priority, diversity, and endemism.