Zoological Journal of the Linnean Society, 2022, **XX**, 1–29. With 10 figures.

Cryptic and widespread: a recipe for taxonomic misidentification in a freshwater crab species (Decapoda: Potamonautidae: *Potamonautes sidneyi*) as evident from species delimitation methods

SAVEL R. DANIELS[1,*], THEO BUSSCHAU[1†], GRAEME GULLACKSEN[2], HANNES MARAIS[3], GAVIN GOUWS[4] and AARON BARNES[1]

[1]*Department of Botany and Zoology, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa*
[2]*Katrinasrust Country Estate, Machadadorp, 1170, South Africa*
[3]*Mpumalanga Tourism and Parks Agency, Aquatic Unit, Lydenburg, 1120, South Africa*
[4]*National Research Foundation - South African Institute for Aquatic Biodiversity, Private Bag 1015, Makhanda, 6140, South Africa*

Received 12 March 2022; revised 5 July 2022; accepted for publication 26 July 2022

We examined the systematics of a ubiquitously distributed southern African freshwater crab, *Potamonautes sidneyi s.l.* species complex. Specimens were subjected to DNA sequence analyses of two mitochondrial loci (16S rRNA + *COI*). We applied three species delimitations methods (ASAP, bGMYC and bPTP) to test their utility in delineating species boundaries in *Potamonautes* and three additional Afrotropical genera (*Liberonautes*, *Nesonautes* and *Seychellum*). The combined mtDNA dataset retrieved five clades. Clade 1 comprised of *P. barbarai*, clade 2 comprised of specimens from the interior of the Great Karoo Basin, sister to *P. sidneyi s.s.* in clade 3. Clade 4 was confined to Eswatini and the Mpumalanga Province of South Africa, and sister to clade 5 that comprised *P. danielsi*. The three species delimitation methods either over- or underestimated the number of species. Phylogenetically, specimens from the Great Karoo Basin (clade 2) were equidistant to *P. sidneyi s.s.* and *P. perlatus*, while the Eswatini and Mpumalanga specimens (clade 4) were sister to *P. danielsi*. Clades 2 and 4 are herein described as ***P. karooensis* sp. nov.** and ***P. valles* sp. nov**., respectively.

ADDITIONAL KEYWORDS: aquatic biodiversity – Decapoda – genetics – systematics.

INTRODUCTION

Historically, taxonomists denoted the presence of ubiquitous taxa using morphological characters. These widespread species were thought to have large, near continuous geographic distributions, presumably lacked habitat specificity and occurred in ecosystems that were highly interconnected and homogenous, like aquatic environments where no contemporary barriers can be observed (Jesse *et al.*, 2010; Phiri & Daniels, 2014; Gouws *et al.*, 2015). However, molecular systematics, fuelled by the revolution in DNA sequence platforms, have revealed an abundance of cryptic diversity, often representing narrow endemic lineages (Jesse *et al.*, 2010; McDonald & Daniels, 2012; Parmakelis *et al.*, 2013; Phiri & Daniels, 2016; Nieto-Montes de Oca *et al.*, 2017; Vitecek *et al.*, 2017; Busschau *et al.*, 2019, 2021; Barnes *et al.*, 2020; Cumberlidge *et al.*, 2021). Frequently, these cryptic lineages appear morphologically similar but are characterized by deep genetic divergences. Cryptic lineages generally occur in taxonomically understudied faunal groups, where subtle morphological characters delineate sister-species and dichotomous species keys are unreliable or outdated (Daniels *et al.*, 2019, 2020a;

*Corresponding author. E-mail: srd@sun.ac.za
†Present address: New York University Abu Dhabi, Saadiyat Island, Abu Dhabi, United Arab Emirates.
[Version of record, published online 2 November 2022; http://zoobank.org/ urn:lsid:zoobank.org:pub:16EA47FF-8FAD-4C4F-9DD9-6255A2B29C9C]

Cumberlidge *et al.*, 2020, 2021; Taylor *et al.*, 2020). These cryptic lineages are now often referred to as molecular operational taxonomic units (MOTUs). A serious limitation to several of these molecular systematic studies is that while authors frequently detect the presence of MOTUs, there is limited description of the novel alpha-taxonomic diversity. Formal taxonomic descriptions remain the hallmark for species to be recognized as distinct evolutionary units. Without the formal description of species, we will continue to underestimate biodiversity and fail to recognize species and habitats in need of conservation.

Species delimitation methods using DNA sequence data are at present widely used in molecular systematic endeavours (Parmakelis *et al.*, 2013; Daniels *et al.*, 2016; Jacobs *et al.*, 2018; Busschau *et al.*, 2019, 2021; Barnes *et al.*, 2020; Ramirez *et al.*, 2022). Theoretically, molecular species delimitation methods offer an unbiased manner to delineate MOTUs that does not require in-depth taxonomic knowledge of groups. Several species delimitation methods are currently in use, each with its advantages and disadvantages (Kapli *et al.*, 2017; Barley *et al.*, 2018). Two main approaches to species delimitation exist, phenetic and coalescent based methods. For example, the assemble species by automatic partitioning (ASAP) method was recently developed to build partitions using single-locus datasets using a distance-based approach (Puillandre *et al.*, 2021). Since ASAP is a newly developed method, it has had limited application in the literature. In contrast, the generalized mixed Yule coalescent model (GMYC) uses a Bayesian species delimitation framework and gene trees to estimate the number of MOTUs in a group. It has been demonstrated that GMYC frequently overestimates the number of MOTUs (Vitecek *et al.*, 2017; Busschau *et al.*, 2019; Barnes *et al.*, 2020; Ellepola *et al.*, 2021; Raphalo *et al.*, 2021a). Multilocus studies are generally preferred to single-locus studies since these provide closer approximations of species trees. Furthermore, these species delimitation methods often render both congruent and incongruent results with respect to the number of species recovered, making it difficult for systematists to decide the number of species present in a species complex (García-Melo *et al.*, 2019). Nevertheless, species delimitation methods provide a well-founded framework for consideration when designating novel taxa, and overall consensus between species delimitations methods provides a point of departure to further delineate or synonymize groups.

Several Afrotropical freshwater crab species of the genus *Potamonautes* MacLeay, 1838, were historically considered to be widespread (Barnard, 1950). However, the application of molecular systematic methods on these presumed widespread species has revealed a wealth of hidden diversity (Daniels, 2011; Phiri & Daniels, 2014, 2016; Peer *et al.*, 2017; Daniels & Klaus, 2018; Cumberlidge *et al.*, 2020, 2021). *Potamonautes sidneyi s.l.* (Rathbun, 1904), is the most ubiquitous of all river crabs in southern Africa (Barnard, 1950; Gouws *et al.*, 2015). The species is thought to be present in eight provinces of South Africa, except the Western Cape (Barnard, 1950; Stewart & Cook, 1998; Peer *et al.*, 2017). *Potamonautes sidneyi s.l.* is also present in neighbouring Eswatini (formerly Swaziland) and Mozambique (Cumberlidge & Daniels, 2008). Barnard (1950) has suggested that there is a transition between *P. sidneyi* and *P. perlatus s.l.* (H. Milne Edwards, 1837) in the Eastern Cape, with clinal variation in morphological features potentially obscuring species boundaries. Further, Phiri & Daniels (2014) described the Gamtoos and Gourits river specimens, previously considered *P. perlatus s.s.*, in the southern part of the Western Cape and in the Eastern Cape provinces as *P. barbarai* Phiri & Daniels, 2014. However, Phiri & Daniels (2014) did not include any specimens from the Great Karoo in their study, rendering the taxonomic provenance of specimens from the latter region unknown. Typically, *P. sidneyi s.l.* inhabits rivers, streams, wetlands and farm dams throughout its distribution range where it occurs from sea level to high-elevation mountains > 1000 m (Barnard, 1950; Cumberlidge & Daniels, 2008). The generalist habitat of *P. sidneyi s.l.* allows the species to live under boulders or to burrow into soft mudbanks along streams, rivers or to live in wetland areas (Barnard, 1950; Cumberlidge & Daniels, 2008; Daniels, pers. obs). Gouws *et al.* (2015) explored the mtDNA lineages in eight *P. sidneyi s.l.* sampling localities along a narrow coastal strip along the Indian Ocean Coastal Belt (IOCB) forest and revealed it to be a species complex for which two clades were retrieved. Clade 1 comprised specimens from the coastal Eastern Cape and southern KwaZulu-Natal provinces, while clade 2 contained the remaining specimens from central KwaZulu-Natal (Gouws *et al.*, 2015). A subsequent taxonomic revision of the *P. sidneyi s.s.* limited the species to the KwaZulu-Natal and Mpumalanga provinces, while the Eastern Cape and southern KwaZulu-Natal clade was described as *P. danielsi* Peer *et al.*, 2017. Peer *et al.* (2017) suggested that *P. danielsi* is also present in Mpumalanga based on morphological comparisons of specimens. However, Daniels *et al.* (2021) demonstrated that *P. danielsi* is absent from the Mpumalanga Province, South Africa. In both the Eastern Cape and Mpumalanga provinces, large inland aquatic regions, such as the Great Karoo and the Great Escarpment, remain unsampled. In fact, two freshwater crab species were recently described from the Eastern Cape and Mpumalanga provinces (Daniels *et al.*, 2021), while a third novel species

from Hogback in the Eastern Cape awaits formal description (N. Peer, pers. comm.). Daniels & Bayliss (2012) reported that specimens from Eswatini were phylogenetically distantly related to *P. sidneyi s.s.* and sister to *P. lividus* Gouws *et al.*, 2001. However, Daniels & Bayliss (2012) were only provided with tissue samples of the freshwater crabs from Eswatini, rendering the identity of the specimens dubious. The Potamonautidae Bott, 1970, has in recent years been the subject of molecular scrutiny, which has resulted in the description of several new species and genera (e.g. Daniels, 2011; Daniels *et al.*, 2015; Daniels & Klaus, 2018; Cumberlidge & Daniels, 2022). The molecular data obtained from these studies, where multiple specimens were sequenced for several genera, provide the ideal opportunity to explore the application and utility of species delimitation methods and their congruence with existing, morphologically based alpha-taxonomy.

During the present study we undertook extensive sampling of *P. sidneyi s.l.* and *P. danielsi* from previously unsampled or poorly sampled regions in South Africa and Eswatini, incorporating most of the distribution ranges of the two species to test for the presence of additional cryptic lineages within the *P. sidneyi s.l.* species complex. We combined this data with data for *P. barbarai* specimens used by Phiri & Daniels (2014) to explore the relationship between the Great Karoo specimens and the latter species complex. We hypothesized that cryptic lineages would be nested within the *P. sidneyi s.l.* species complex, considering its wide geographic distribution. To test for the presence of novel lineages in the *P. sidneyi s.l.* species complex we employed three species delineating methods (ASAP, bGYMC and bPTP) using mtDNA sequence data for the *Potamonautes*. In addition, we used mtDNA sequence data (for the cytochrome *c* oxidase I subunit) from three Afrotropical freshwater crab genera (*Liberonautes* Bott, 1955, *Nesonautes* Cumberlidge & Daniels, 2022 and *Seychellum* Ng *et al.*, 1995), where fine-scale sampling had been undertaken and cryptic lineages observed, to test the application of the three aforementioned delimitation methods in retrieving the currently recognized species diversity (Cumberlidge & Daniels, 2014, 2019; Daniels *et al.*, 2016). We hypothesized that the three species delimitation methods would, to some degree, overestimate diversity relative to the known taxonomic diversity. Furthermore, we placed the two novel *Potamonautes* species in a divergence-time framework with other southern African freshwater crabs to date speciation and to understand the spatiotemporal mechanisms that might have been central to cladogenesis. We also describe the two novel freshwater crab species from southern Africa revealed in the present study.

MATERIAL AND METHODS

Sample collection

Freshwater crabs were hand-collected from under rocks in boulder-strewn rivers, streams and wetlands throughout the known distribution ranges of the two focal species (Table 1; Fig. 1). For *Potamonautes sidneyi s.s.*, we sampled 30 localities in Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga and the Northern Cape provinces of South Africa, while one locality was sampled in Eswatini. For *P. danielsi*, we sampled eight localities in southern KwaZulu-Natal and the coastal and adjacent interior of the Eastern Cape Province. In addition, four of the new *P. karooensis* localities were sampled in the Eastern Cape, South Africa, while two localities for the new *P. valles* were sampled in the Mpumalanga provinces, South Africa, as well as a single locality in Eswatini. The Papkuilsfontein locality of *P. barbarai* was combined with specimens from 13 conspecific populations sequenced by Daniels *et al.* (2006) (Fig. 1). Specimens were killed by freezing, labelled and placed into jars filled with absolute ethanol. A handheld GPS was used to record latitude and longitude at each sample locality. Photographic images of live freshwater crabs were captured with the use of a Canon EOS 90D camera and a Sigma 105 mm f2.8 Macro USM lens.

DNA extraction, PCR and sequencing

Muscle tissue, extracted from walking legs, was subjected to DNA extraction using a Nucleospin kit (Macherey-Nagel, Duren, Germany), following the manufacturers' protocol. For the phylogeographic aspect of the study, we focused on sequencing the 16S rRNA locus. This locus has been widely used in phylogeographic studied of decapods, including freshwater crabs. The 16S rRNA locus was sequenced for all *P. sidneyi s.l.* and *P. danielsi* specimens. We combined the genetic data from the current study with representative mtDNA sequences for *P. barbarai* (Phiri & Daniels, 2014). We combined the newly generated DNA sequence data with that from five small-scale studies on the latter two species (Daniels & Bayliss, 2012; Daniels *et al.*, 2014, 2019; Gouws *et al.*, 2015; Daniels, 2017). The 16S rRNA topology was used as a guide tree from which we selected the major clades and sequenced these specimens for the cytochrome *c* oxidase I subunit (*COI*) locus. Subsequently, we combined the two mtDNA datasets (16S rRNA + *COI*) for which both loci were sequenced and used this data

Table 1. List of the five freshwater crab species from 59 localities, including two new species collected from South Africa and Eswatini during the present study and combined with DNA sequence data from five earlier studies (Daniels & Bayliss, 2012; Daniels *et al.*, 2014, 2019; Gouws *et al.*, 2015; Daniels, 2017). Data for 13 sample localities of *P. barbarai* were obtained from Daniels *et al.* (2006). The locality number corresponds to the map (Fig. 1). Nature reserves are denoted with NR

Locality Number	Locality	Province, Country	Species	*N*	S	E	16S rRNA	*COI*	Reference study
1	Dwesa NR	Eastern Cape, South Africa	*P. danielsi*	2	32°15′86.8″	28°51′47.6″	2	2	Present study
2	Thorn River	Eastern Cape, South Africa	*P. danielsi*	3	32°19′27.2″	27°09′52.2	3	3	Present study
3	Mbotyi	Eastern Cape, South Africa	*P. danielsi*	8	31°28′35.9″	29°42′7.16″	8	7	Daniels (2017)
4	Fort Fordyce NR	Eastern Cape, South Africa	*P. danielsi*	2	32°41′23.2″	26°29′94.5″	2	2	Present study
5	Hogsback	Eastern Cape, South Africa	*P. danielsi*	1	32°36′02.6″	26°55′85.2″	1	1	Present study
6	Oribi Gorge NR	KwaZulu-Natal, South Africa	*P. danielsi*	4 + 3	30°42′37.6″	30°16′21.1″	5	5	Present study; Gouws *et al.* (2015)
7	Mtamvuna	KwaZulu-Natal, South Africa	*P. danielsi*	3	31°03′31.6″	30°10′26.1″	1	3	Gouws *et al.* (2015)
8	Sani Pass	KwaZulu-Natal, South Africa	*P. danielsi*	1	29°37′56.0″	29°25′08.2″	1	1	Present study
9	Asante Sana	Eastern Cape, South Africa	*P. karooensis* sp. nov	4	32°16′12.3″	24°57′25.4″	4	5	Present study
10	Langfontein farm	Eastern Cape, South Africa	*P. karooensis* sp. nov	3	32°11′81.2″	24°09′90.5″	3	3	Present study
11	Ouberg Pass	Eastern Cape, South Africa	*P. karooensis* sp. nov	4	32°04′09.3″	24°21′42.0″	4	4	Present study
12	Erasmuskloof farm	Eastern Cape, South Africa	*P. karooensis* sp. nov	4	32°11′44.0″	24°47′44.0″	4	4	Present study
13	Blood River	KwaZulu-Natal, South Africa	*P. sidneyi*	4	28°06′19.0″	30°32′30.0″	4	0	Present study
14	Buffels River	KwaZulu-Natal, South Africa	*P. sidneyi*	3	28°42′59.0″	30°38′30.0″	3	0	Present study
15	Cathedral Peak NR	KwaZulu-Natal, South Africa	*P. sidneyi*	6	28°56′38.3″	29°13′50.4″	6	0	Present study
16	Ngoye Forest	KwaZulu-Natal, South Africa	*P. sidneyi*	1	28°52′10.2″	31°41′22.9″	1	1	Daniels *et al.* (2019)
17	Nkandla Forest	KwaZulu-Natal, South Africa	*P. sidneyi*	4	28°44′10.4″	31°08′05.7″	4	4	Daniels *et al.* (2019)

Table 1. Continued

Locality Number	Locality	Province, Country	Species	*N*	S	E	16S rRNA	*COI*	Reference study
18	Hlulhuwe	KwaZulu-Natal, South Africa	*P. sidneyi*	5 + 1	28°02′20.0″	32°05′10.0″	6	3	Present study; Gouws *et al.* (2015)
19	Lake Sibaya	KwaZulu-Natal, South Africa	*P. sidneyi*	1	27°21′50.0″	32°31′35.0″	1	1	Gouws *et al.* (2015)
20	Entumeni	KwaZulu-Natal, South Africa	*P. sidneyi*	1	28°53′24.6″	31°18′47.4″	1	0	Gouws *et al.* (2015)
21	Mahai	KwaZulu-Natal, South Africa	*P. sidneyi*	5	28°41′17.7″	28°56′51.0″	5	0	Present study
22	Mpophme	KwaZulu-Natal, South Africa	*P. sidneyi*	3	29°34′55.9″	30°11′10.2″	0	1	Gouws *et al.* (2015)
23	Amagoda	KwaZulu-Natal, South Africa	*P. sidneyi*	5	27°46′30.0″	30°46′0.10″	5	0	Present study
24	Siqoba, Vryheid	KwaZulu-Natal, South Africa	*P. sidneyi*	5	27°46′01.0″	30°48′00.0″	5	0	Present study
25	Mhlanga	KwaZulu-Natal, South Africa	*P. sidneyi*	3	29°43′42.4″	31°04′10.6″	1	1	Gouws *et al.* (2015)
26	Kosi Bay	KwaZulu-Natal, South Africa	*P. sidneyi*	2	26°58′14.7″	32°48′21.4″	2	2	Present study
27	Manguzi Forest	KwaZulu-Natal, South Africa	*P. sidneyi*	4	27°01′03.6″	32°43′32.1″	4	4	Present study
28	Hlatikulu Forest	KwaZulu-Natal, South Africa	*P. sidneyi*	4	27°19′54.8″	31°59′07.2″	4	4	Present study
29	Tchiombedi river	Limpopo, South Africa	*P. sidneyi*	4	22°45′42.0″	30°28′50.9″	4	0	Present study
30	Mukhasa, Cycad NR	Limpopo, South Africa	*P. sidneyi*	3	24°03′54.8″	30°50′50.6″	3	0	Present study
31	Overwaal	Gauteng, South Africa	*P. sidneyi*	5	26°42′37.9″	27°51′39.3″	5	0	Present study
32	Paul Kruger Bridge	Mpumalanga, South Africa	*P. sidneyi*	5	26°46′17.6″	29°55′19.6″	5	0	Present study
33	Amersfoot	Mpumalanga, South Africa	*P. sidneyi*	3	26°28′30.2″	29°47′52.3′	3	0	Present study
34	Vaal B	Mpumalanga, South Africa	*P. sidneyi*	3	26°46′46.0″	29°55′05.0″	3	0	Present study
35	Warburton	Mpumalanga, South Africa	*P. sidneyi*	4	25°58′57.0″	29°44′04.9″	4	0	Present study
36	Wakkerstroom	Mpumalanga, South Africa	*P. sidneyi*	4	27°21′33.7″	30°08′44.4″	4	0	Present study

Table 1. Continued

Locality Number	Locality	Province, Country	Species	*N*	S	E	16S rRNA	*COI*	Reference study
37	Lake Chrissiemeer	Mpumalanga, South Africa	*P. sidneyi*	1	26°17′55.3‘	30°13′41.4″	1	1	Daniels *et al.* (2014)
38	Verloren Vallei NR	Mpumalanga, South Africa	*P. sidneyi*	14	25°20′33.3″	30°07′54.6″	14	14	Daniels *et al.* (2014)
39	Iona farm	Mpumalanga, South Africa	*P. sidneyi*	3	26°13′77.7‘	30°16′66.7″	3	3	Daniels *et al.* (2014)
40	Blyde River Canyon NR (BRCNR)	Mpumalanga, South Africa	*P. sidneyi*	1	24°42′36.3″	30°54′11.6″	1	1	Present study
41	Sabie	Mpumalanga, South Africa	*P. sidneyi*	3	25°05′44.4″	30°45′58.7″	3	0	Present study
42	Upington	Northern Cape, South Africa	*P. sidneyi*	5	28°27′15.5″	21°14′53.1″	5	5	Present study
43	Papkuilsfontein	Northern Cape, South Africa	*P. barbarai*	1	31°33′1.94″	19°08′07.3″	1	1	Present study
44	Sterkspruit	Mpumalanga, South Africa	*P. valles* sp. nov.	3	26°40′40.0″	30°07′29.0″	3	0	Present study
45	Blyde River Canyon NR	Mpumalanga, South Africa	*P. valles* sp. nov.	5	24°40′14.8″	30°52′34.1″	5	5	Present study
46	Mbabane district	Hohohho, Eswatini	*P. valles* sp. nov.	3 + 2	26°12′24.1″	31°07′59.6″	3	5	Present study; Daniels & Bayliss (2012)
47	Vette River	Western Cape, South Africa	*P. barbarai*	1	34° 01′ 4.8″	21°13′ 8.9″	1	1	Daniels *et al.* (2006)
48	Huis River	Western Cape, South Africa	*P. barbarai*	1	33° 30′07.8″	21°36′02.9″	1	1	Daniels *et al.* (2006)
49	Prince Albert	Western Cape, South Africa	*P. barbarai*	1	33°07′00.0″	21°55′13.4″	1	1	Daniels *et al.* (2006)
50	Groot River	Western Cape, South Africa	*P. barbarai*	1	33°40′15.0″	21°10′4.70″	1	1	Daniels *et al.* (2006)
51	Dwyka River	Western Cape, South Africa	*P. barbarai*	1	33°05′01.7″	21°34′12.9″	1	1	Daniels *et al.* (2006)
52	Hankey	Eastern Cape, South Africa	*P. barbarai*	1	33°50′ 00.0″	24°52′11.7″	1	1	Daniels *et al.* (2006)
53	Patensie	Eastern Cape, South Africa	*P. barbarai*	1	33°35′16.3″	24° 46′10.7″	1	1	Daniels *et al.* (2006)
54	Poortjies	Eastern Cape, South Africa	*P. barbarai*	1	33°39′10.7″	24° 32′ 03.5″	1	1	Daniels *et al.* (2006)
55	Bosdorp	Eastern Cape, South Africa	*P. barbarai*	1	33°39′10.8″	21°26′00.6″	1	1	Daniels *et al.* (2006)

Table 1. Continued

Locality Number	Locality	Province, Country	Species	*N*	S	E	16S rRNA	*COI*	Reference study
56	Nels River	Western Cape, South Africa	*P. barbarai*	1	33°29′10.8″	21°26′ 06.6″	1	1	Daniels *et al.* (2006)
57	Vlei River	Western Cape, South Africa	*P. barbarai*	1	33°33′ 6.6″	21 53′05.5″	1	1	Daniels *et al.* (2006)
58	Andrieskraal	Eastern Cape, South Africa	*P. barbarai*	1	33°44′15.5″	24°38′02.5″	1	1	Daniels *et al.* (2006)
59	Kleinplaats	Eastern Cape, South Africa	*P. barbarai*	1	33°38′12.9″	24°27′14.2″	1	1	Daniels *et al.* (2006)

to run three selected species delimitation methods. Finally, the 12S rRNA locus was sequenced for the two potential novel lineages and all three mtDNA loci were placed in a phylogenetic context with the described southern African *Potamonautes* (Phiri & Daniels, 2014, 2016; Daniels *et al.*, 2015, 2019; Gouws *et al.*, 2015; Wood & Daniels, 2016; Daniels & Klaus, 2018). Primer pairs for the three mitochondrial loci, polymerase chain reaction (PCR) conditions for amplification and DNA sequencing protocols are outlined in Daniels *et al.* (2015). Sequences of each of the three mitochondrial gene regions were downloaded from GenBank for all the described southern African freshwater crab species (Daniels & Bayliss, 2012; Daniels *et al.*, 2014, 2019; 2020a, b; Phiri & Daniels, 2014; Wood & Daniels, 2016; Daniels, 2017). We used five *Maritimonautes* Cumberlidge & Daniels, 2022 species, *M. calcaratus* (Gordon, 1929), *M. choloensis* (Chace, 1953), *M. licoensis* (Daniels *et al.*, 2020a), *M. namuliensis* (Daniels & Bayliss, 2012) and *M. obesus* (A. Milne-Edwards, 1868), as outgroups.

Phylogenetic analyses and divergence-time estimation

Forward and reverse DNA strands were used to compute a consensus sequence and to check for base ambiguities using SEQUENCE NAVIGATOR (Applied Biosystems, Foster City, California, USA). Sequence alignment was computed using CLUSTAL X v.2.1 (Thompson *et al.*, 1997). Using statistical parsimony in TCS 1.06 (Clement *et al.*, 2000) a haplotype network was constructed from the 16S rRNA sequence data for *P. sidneyi s.s.*, *P. barbarai* and *P. danielsi* data. Maximum likelihood (ML) and Bayesian inference (BI) were used to infer phylogenetic relationships for the 16S rRNA only data, the *COI* + 16S rRNA dataset and the three mtDNA loci (12S rRNA, 16S rRNA and *COI*) combined. For the ML analyses, the IQ-Tree web server (v.1.4.3, http://iqtree.cibiv.univie.ac.at/, Trifinopoulos *et al.*, 2016) was used to select for the optimal DNA substitution model and the best-fit likelihood score, which was chosen using the Akaike information criterion (AIC) (Akaike, 1973). For the BI analyses, the AIC (Akaike, 1973) was used to select the optimal DNA substitution model in jModelTest2 v.2.1.6 (Posada & Crandall, 1998) on XSEDE through the CIPRES Science Gateway (Miller *et al.*, 2010). The ML tree inference was performed using the IQ-Tree web server (v.1.4.3, http://iqtree.cibiv.univie.ac.at/, Trifinopoulos *et al.*, 2016). Bootstrap values > 75% were deemed as sufficient support for nodes.

Bayesian analyses were conducted on CIPRES (v.3.3, Miller *et al.*, 2010) using MrBayes v.2.7a (Ronquist *et al.*, 2012) on XSEDE. Each analysis comprised of one run of four chains for 50 million

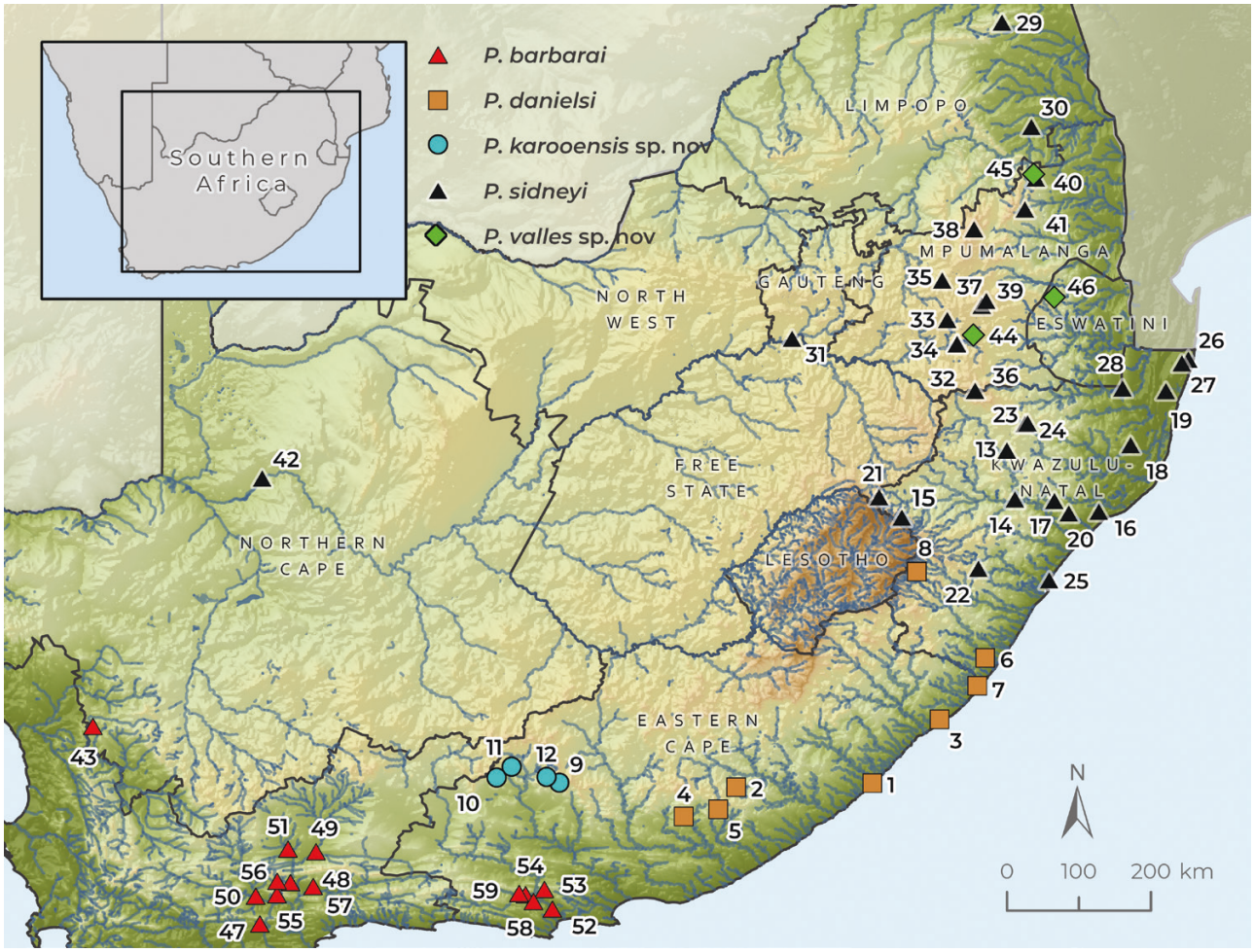

Figure 1. A map of southern Africa (South Africa and Eswatini) showing the sites where freshwater crabs were collected during the present study. The 30 *Potamonautes sidneyi s.s.* sample localities are represented by solid, black triangles; the eight *P. danielsi* sample localities are represented by brown squares, while the four sample localities for *P. karooensis* sp. nov. are represented by a turquoise circle and three sample localities for *P. valles* sp. nov., are represented by a green diamond. The Papkuilsfontein locality of *P. barbarai* was combined with specimens from 13 conspecific populations sequenced by Daniels *et al.* (2006) and represented by a red triangle. The sample numbers correspond to the 59 localities in Table 1.

generations, sampling every 1000 generations using default parameters. A random tree was selected for the start of each chain. Burn-in was included in the command block and set to 20% as discerned in TRACER v.1.6 (Rambaut *et al.*, 2014), along with ensuring an effective sampling size (ESS) of values > 200 for all parameters. After the burn-in trees were discarded a 50% majority rule consensus tree was generated from the trees retained. The percentage time a node was recovered provided the posterior probability (PP) for each node. Posterior probabilities (PP) < 0.95 were regarded as statistically poorly supported. Uncorrected '*p*'-distances were calculated among and within putative species in the *P. sidneyi* species complex for the *COI* locus in PAUP v.4.0b10 (Swofford, 2002).

Divergence-time estimations were conducted for the combined mtDNA dataset (16S rRNA, 12S rRNA + *COI*) using a Bayesian framework, which makes use of a probabilistic model to define the molecular sequence divergence of lineages, which further makes use of the Markov chain Monte Carlo (MCMC) method to estimate clade ages. Mutation rates for each locus were inferred from a fossil-calibrated phylogeny of the Potamonautidae (Daniels, 2011; Daniels *et al.*, 2015; Daniels & Klaus, 2018): 0.81% per Myr for the rRNA loci (SD = 0.0013; linked clock models), 2.85% per Myr (SD = 0.005) for the *COI* locus. A Yule tree prior was applied. The maximum clade credibility tree was determined and annotated in TreeAnnotator v.2.4.1 (part of the BEAST package) after removal of 20% of the trees as burn-in. A strict molecular clock was used

(Drummond *et al.*, 2006), and the analyses were run using the program BEAST2 v.2.4.8 (Drummond & Rambaut, 2007).

A multiple coalescent model was used (Heled & Drummond, 2010). jModelTest2 v.2.1.6 (Posada & Crandall, 1998) on XSEDE through CIPRES (Miller *et al.*, 2010) was used to determine the parameters and substitution models for each locus. One hundred million generations were run for ten independent MCMC chains, with sampling performed every 5000 generations. After an appropriate burn-in, the convergence of the ten combined chains was determined by ESS for each parameter in TRACER (v.2.4.8, https://beast.community/logcombiner, Rambaut *et al.*, 2014). Trees were assessed and a maximum clade credibility tree was formulated using TreeAnnotator. The program FIGTREE v.1.4.3 (Rambaut, 2009) was used to formulate a chronogram.

SPECIES DELIMITATION USING ASAP, BPTP AND BGMYC

Candidate lineages were identified for further analyses using the *COI* phylogenetic topology. We used *COI* data for four genera for which we had multiple samples available per species to test the use of the species delimitation methods employed. These included *COI* data for *Liberonautes*, *Nesonautes* and *Seychellum*, from three previous studies (Daniels, 2011; Daniels *et al.*, 2016; Daniels & Klaus, 2018). Since the taxonomy of the three aforementioned genera is stable or has undergone recent revision, this allowed us to test the morphologically defined species designations (Cumberlidge & Daniels, 2013, 2019). These three genera represent three distinct evolutionary scenarios: in *Liberonautes*, at least two MOTUs are present that are sister to *L. rubigimanus* Cumberlidge & Sachs, 1989; in *Seychellum*, three allospecies that are geographically distinct and confined to island clusters are known; in *Nesonautes*, the two species are the result of sympatric speciation. For *Potamonautes*, we combined *COI* data for South African species including *P. barnardi* Phiri & Daniels, 2014, *P. barbarai*, *P. brincki* (Bott, 1960), *P. depressus* (Krauss, 1843), *P. clarus* Gouws *et al.*, 2000, *P. flavusjo* Daniels *et al.*, 2014, *P. lividus*, *P. parvicorpus* Daniels *et al.*, 2001, *P. ntendekaensis* Daniels *et al.*, 2019 and *P. ngoyensis* Daniels *et al.*, 2019, *P. perlatus* and *P. tuerkayi* Wood & Daniels, 2016 (Daniels & Bayliss, 2012; Phiri & Daniels, 2014, 2016; Wood & Daniels, 2016; Daniels *et al.*, 2019, 2020) with representative sequences for *P. sidneyi s.l.* and *P. danielsi* generated during the current study, including data from four earlier studies (Daniels & Bayliss, 2012; Daniels *et al.*, 2014, 2019; Daniels, 2017).

We used the newly developed assemble species by automatic partitioning (ASAP) (Puillandre *et al.*, 2021). The method uses genetic distances to hierarchically cluster species partitions (https://bioinfo.mnhn.fr/abi/public/asap). ASAP first assigns a probability that each new clustering is a new species and then computes the relative width of the barcode gap of a partition in relation to the previous partitions. These metrics are combined into an ASAP score to rank all partitions detected in the analyses. Since ASAP is an exploratory method that does not consider the evolutionary history among sequences, we report the first two partitions ranked by ASAP score, using *p*-distances and the default setting splitting groups below probability < 0.01.

A Bayesian implementation of the Poison tree processes (bPTP) was run on the online bPTP web server (https://species.h-its.org/ptp/) for its ability to delimit species without a priori knowledge of population parameters (Zhang *et al.*, 2013). The analysis was run for 500 000 MCMC generations with a thinning value = 100 and burn-in = 0.20. The convergence of the MCMC chain was visually confirmed as recommended by Zhang *et al.* (2013). The latter parameters were utilized for each of the four Afrotropical freshwater crab genera. We also employed a Bayesian implementation of the GMYC model using the R package bGMYC (Reid & Carstens, 2012). To account for error in phylogenetic estimation, 500 post-burn-in trees were randomly selected for analysis. A Markov chain was run for 50 000 generations, sampling the chain every 1000th generation and 4000 generations were discarded as burn-in. A uniform prior for the number of species was applied to each genus with a lower bound of one and varied upper bounds set to the number of sequences in the analyses. For *Potamonautes*, an upper bound of 319 was implemented, for both *Liberonautes* and *Nesonautes*, an upper bound of 102 was implemented and, finally, for *Seychellum* an upper bound of 83 was implemented. Convergence was assessed visually by examining the performance of the chain. The 'check rates' function was used to determine the rate of branching of the coalescent model relative to that of the mixed Yule model. The latter parameters were utilized for each of the four genera under investigation during the bGMYC analyses. Outgroups were removed for all three delimitation methods.

MORPHOLOGY

For the taxonomic description of newly discovered species, characters for male and female specimens from possible new lineages were considered separately, since freshwater crabs exhibit sexual dimorphism. The following measurements were taken with digital callipers: carapace length (CL); the carapace width

at widest point (CWW); the width of the posterior margin of the carapace (CWP); the distance between the postfrontal crest and the anterior margin of the carapace (PFCD); the frontal width, measured between the medial margins of the orbits (FW); the distance between the exorbital teeth (CWA); the carapace height (CH); the length and width of the merus of pereiopods 2 and 5 (PML and PMW, respectively), the length of the propodus of the major cheliped (MCPL), as well as the major cheliped dactylus length (MCDL). All measurements are given in millimetres (mm). Specimens of the two new species were deposited in the South African Museum of Natural History, Iziko Museums of Cape Town (SAM). In addition, the major and minor chelipeds of the new species were photographed with the aforementioned camera setup. The structure of gonopods 1 and 2, and the maxillipeds of the two new species were compared.

RESULTS

Combined mtDNA topology for the *Potamonautes sidneyi* species complex

For the 16S rRNA locus, we amplified a 431 base pair (bp) fragment for 123 new specimens and deposited in GenBank (accession numbers for *P. karooensis*, OL685395–OL685398, OL685401–OL685411; *P. sidneyi s.s.*, OL685412–OL685417, OL685423–OL685238, OL685442–OL68503; *P. danielsi*, OL685356–OL685368; *P. valles*, OL685399–OL685400, OL685418–OL685422, OL685439–OL685440; *P. barbarai*, OL685504); a further 151 novel sequences from five previous studies, and 25 sequences of *P. barbarai* resulted in a total of 176 sequences. The DNA substitution model for 16S rRNA was TIM2+I+G. The tree topologies derived from the BI and ML analyses retrieved a monophyletic *P. sidneyi s.l.* species complex (> 0.95 PP/ > 75%). However, nodal relationships were poorly supported (Supporting Information, Fig. S1) and the analyses of the 16S rRNA alone were of limited utility to resolve relationships among the species complex. Basal in the tree topology was a clade exclusive to the Great Karoo Basin in the Eastern Cape Province, South Africa. Second, a large *P. sidneyi s.l.* grouping, comprising specimens from KwaZulu-Natal, Gauteng, Mpumalanga, Limpopo and the Northern Cape provinces, was retrieved with *P. barbarai* nested within the latter complex. In addition, a clade of *P. danielsi* from the southern KwaZulu-Natal and the coastal and adjacent interior of the Eastern Cape was sister to a clade comprising specimens from eastern Mpumalanga Province (Muilhuis at Blyde River Canyon Nature Reserve and Sterkspruit) in South Africa and specimens from Eswatini were statistically well supported (> 0.95 PP/ > 75%). The 16S rRNA haplotype network (Supporting Information, Fig. S2) comprised 51 haplotypes, and failed to yield any distinct phylogeographic groupings, as there was sharing of haplotypes between haploclusters, suggesting that the marker was of limited phylogeographic utility.

For the *COI* locus, a 583 bp fragment was amplified for 53 new specimens, combined with 43 *COI* sequences of *P. sidneyi s.l.* from five previous studies. The *COI* data were combined with the corresponding 16S rRNA data for a total of 96 specimens, plus the corresponding two markers for *P. barbarai*. The DNA substitution models for the 16S rRNA and *COI* loci were TIM2+I+G and TVM+I+G, respectively. Novel sequences were submitted to GenBank (accession numbers OL660567–OL660572, OL660578–OL660587 for *P. sidneyi s.s.*; OL685173–OL685185 for *P. danielsi*; OL660552–OL660566 for *P. karooensis*; for *P. valles*, OL660549–OL660551, OL660573–OL660577; for *P. barbarai* OL660588). The combined mtDNA dataset (16S rRNA + *COI*) comprised 1015 bp. The tree topologies derived from both the BI and ML analyses were near identical; hence only the ML topology is shown (Fig. 2). Five statistically, well-supported clades were retrieved (> 0.95 PP/ > 75%). Clade 1 contained *P. barbarai* exclusively and comprised specimens from the Gamtoos and Gourits rivers in the Eastern Cape and a single specimen from Papkuilfontein in the Northern Cape provinces. Clade 2 was exclusive to the Great Karoo Basin interior of the Eastern Cape Province and contained samples from Erasmuskloof, Ouberg Pass, Asante Sana and Langfontein. Clade 3 was comprised exclusively of the *P. sidneyi s.s.* and is widespread in three provinces: KwaZulu-Natal (Ngoye Forest, Manguzi Forest, Hlatikulu Forest, Kosi Bay, Mpophomeni, Lake Sibaya, Hluhluwe and Nkandla Forest), Mpumalanga [Blyde River Canyon Nature Reserve (BRCNR), Verloren Vallei Nature Reserve, Iona Farm and Lake Chrissiesmeer] and the Northern Cape (Upington). *Potamonautes sidneyi s.s.* (clade 3) was sister to clades 4 and 5 (Fig. 2). Clade 4 comprised specimens from the Muilhuis section of the BRCNR in the Mpumalanga Province, South Africa specimens and specimens from near Mbabane in Eswatini. Clade 5 was comprised exclusively of *P. danielsi* specimens collected from southern KwaZulu-Natal (Sani Pass, Oribi Gorge Nature Reserve, Mhlanga and Mtamvuna) and the adjacent coastal (Dwesa Nature Reserve and Mbotyi) and the interior (Hogsback, Fort Fordyce Nature Reserve and Thorn River) of the Eastern Cape Province.

The minimum uncorrected interspecific *COI* '*p*'-distance between clades 2 and 3 (the latter being comprised of *P. sidneyi s.s.*) was 7.33%, while the uncorrected *COI* '*p*'-distance between clades 4 and 5 (the latter being comprised of *P. danielsi* exclusively) was 10.29%. The maximum uncorrected '*p*' intraspecific *COI* distance within clade 2 was 1.02%, within clade 3

Figure 2. Maximum likelihood phylogenetic tree topology derived from the combined 16S rRNA + *COI* sequence data, demonstrating the evolutionary relationships within the *P. sidneyi s.l.* species complex. Statistical support for nodes is provided as posterior probability values above nodes (> 0.95 PP) and bootstrap values below nodes (> 75%). An * or #

it was 6.90%, within clade 4 it was 2.23% and, finally, within clade 5 it was 5.14%. Clades 2 and 4 are here described as the new species *P. karooensis* and *P. valles*, respectively.

Species delimitation using ASAP, bGMYC and bPTP

The results of the three species delimitation methods were largely incongruent for the four Afrotropical freshwater crab genera (Fig. 3A–D; Table 2). For *Potamonautes*, the first two partitions retrieved by ASAP differed only slightly in the amount of putative species identified, with the first and second partitions retrieving 15 and 17 putative species, respectively. Partition two recognized more genetic structuring within clades, especially for those in which certain specimens falling basal relative to the remainder of the clade. As for the focal *Potamonautes* species in the present study, both partitions retrieved *P. sidneyi s.s.*, whereas partition one clustered *P. barbarai*, *P. karooensis* and *P. perlatus* as a single putative species. Further, both partitions clustered *P. barbarai* and *P. karooensis* as a single putative species. *Potamonautes danielsi* and *P. valles* were retrieved as distinct putative species by both partitions (Fig. 3A). Within the genus *Liberonautes*, partition one retrieved 12 putative species, while partition two retrieved five putative species, the same number of lineages suggested by Daniels *et al.* (2016) (Fig. 3B). Within *Nesonautes*, partition one identified eight putative species, while partition two identified two putative species; the latter is identical to the number of described species (Fig. 3C). In *Seychellum*, partition one identified two species; however, partition two overestimated the number of species present, with 24 putative species being retrieved (Fig. 3D).

In order to delimit species, the output of the Bayesian implementation of the bGMYC analysis requires an established threshold at which individuals could be considered conspecific. Due to the lack of inclusion of outgroups outside of the focal genera, the analysis was more sensitive to population-level variation. Therefore, a conservative threshold of $P > 0.5$ was chosen for each of the four genera based on its relative accuracy in comparison to each of the established taxonomies. The bGMYC analysis retrieved 28 putative species for *Potamonautes*, two species for *Nesonautes*, eight species in *Liberonautes* and five species for *Seychellum*. The latter results overestimated the number of species within *Liberonautes*, *Seychellum* and *Potamonautes*, but recognized three of the focal species within *Potamonautes*, *P. barbarai*, *P. karooensis* and *P. valles* as distinct species. Both *P. danielsi* and *P. sidneyi s.s.* showed the same pattern of intraspecific clades being retrieved as distinct species.

The bPTP analysis overestimated the number of species for *Potamonautes*, retrieving 53 putative species. However, two of the five focal species, *P. karooensis* and *P. valles*, were supported as distinct (Fig. 3A). The remaining three focal species, *P. sidneyi s.s.*, *P. danielsi* and *P. barbarai*, were oversplit with intraspecific clades and intraspecifically divergent specimens being retrieved as distinct species. Further oversplitting throughout the topology could be attributed to the retrieval of random specimens as distinct putative species, as seen in *P. barbarai*, a result that could not be attributed to genetic variation based on both the phylogenetic analyses and the other species delimitation methods employed. The bPTP analysis exhibited a similar trend of overestimation for the remaining three genera, retrieving 14 putative species for *Liberonautes*, 22 putative species for *Nesonautes* and 17 putative species for *Seychellum*. Among these three genera, the bPTP analysis assigned putative species statuses to a number of consecutive individual specimens from intraspecific clades.

Phylogenetic placement of two novel lineages among the Southern African *Potamonautes* and divergence-time estimation

The southern African *Potamonautes* was retrieved as monophyletic (Fig. 4). The small-bodied mountain-stream species formed a distinct clade. Among these three Great Escarpment mountain species, *P. baziya* Daniels *et al.*, 2021, *P. clarus* and *P. depressus* were a sister-clade to the five species from the Cape Fold Mountains, *Potamonautes* (from Hogsback) was sister to *P. parvispina* Stewart, 1997 from the Cederberg Mountains, with these two species sister to *P. parvicorpus*, *P. brincki* and *P. tuerkayi*. The following clade contained the coastally distributed species, with *P. valles*, and *P. danielsi* as successive basal taxa to *P. isimangaliso* Peer & Gouws, 2015 and *P. lividus*. This coastal clade was sister to a clade of subtropical/temperate species containing most of the recently described large-bodied riverine species. In the subtropical/temperate clade, *P. dentatus* Stewart *et al.*, 1995 was sister to *P. mhlophe* Daniels, 2017,

denotes nodal relationships that were not supported (< 0.95 PP/ < 75%). *Potamonautes sidneyi s.s.* (clade 3) localities are marked with a dark blue triangle, while *P. danielsi* (clade 5) localities are marked by an orange square. The two new species, *P. karooensis*, (clade 2) and *P. valles* (clade 4), are marked by a light-blue circle and a green diamond, respectively. Specimens of *P. barbarai* are confined to clade 1.

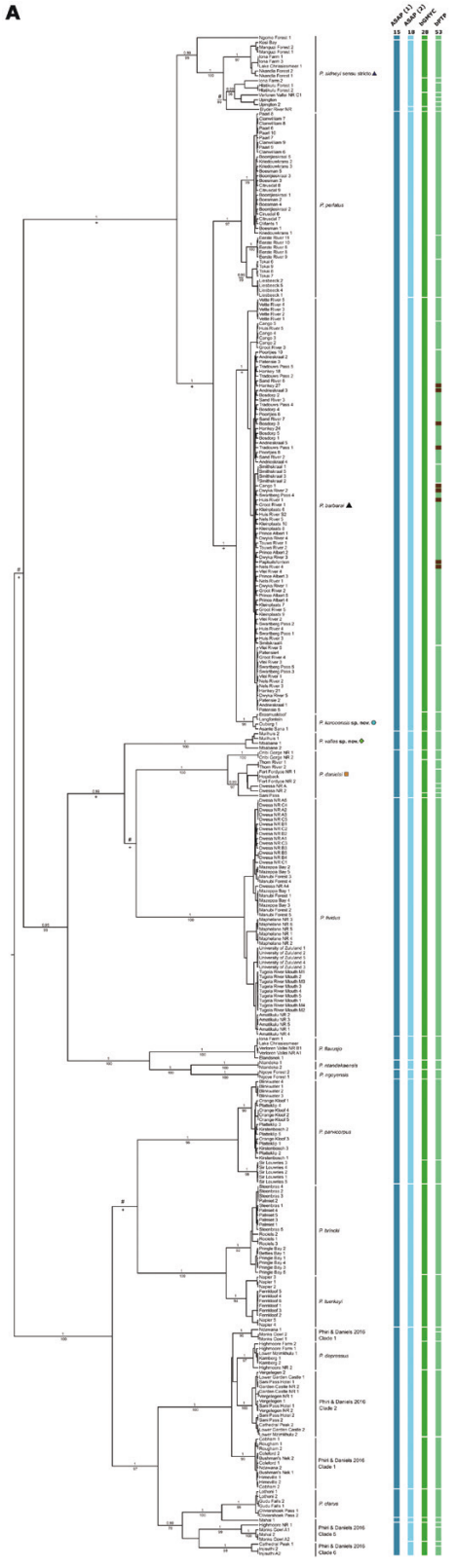

Figure 3. Ultrametric BEAST tree phylogeny of the *COI* sequence data for the four Afrotropical genera: A, *Potamonautes*; B, *Liberonautes*; C, *Nesonautes*; D, *Seychellum*. The four vertical-coloured bars represent alternative taxonomies with each segment representing distinct species according to the respective delimitation method employed. The first two bars represent results from the ASAP method (partitions one and two). Statistical support for nodes is derived from the species tree methods bGMYC (above branch, posterior probability values derived from the BEAST analysis) and bPTP (below branch as bootstrap values derived from the maximum likelihood analysis). Non-supported nodal relationships (< 0.95 PP/ < 75%) are shown by their relevant symbols: (#) bGMYC and bPTP. Dark brown segments within the PTP bars represent the retrieval of individual specimens as putative species.

with this clade basal to a larger clade of three forest-dwelling taxa, *P. mariepskoppie* Daniels *et al.*, 2021, *P. ngoyensis* and *P. ntendekaensis*, which was sister to a clade containing *P. flavusjo*, *P. mulanjeesis* Daniels & Bayliss, 2012, *P. gorongosa* Cumberlidge *et al.*, 2017 and *P. mutareensis* Phiri & Daniels, 2013. In the large-bodied riverine crab clade, the species with spines on the anterolateral margin, *P. bayonianus* (Brito-Capello, 1864), was sister to *P. warreni* (Calman, 1918) and *P. unispinus* Stewart & Cook, 1998, which together were sister to those species lacking dentition on the anterolateral carapace margins; the latter group comprised successive sisters *P. sidneyi*, *P. karooensis*, *P. perlatus* and *P. barnardi* to *P. barbarai* and *P. granularis* Daniels *et al.*, 1998.

Divergence-time estimates within the monophyletic southern African freshwater crabs suggest that cladogenesis initiated 10.35 million years ago (Mya) (95% HPD 14.18–7.39 Mya) during the middle Miocene (Fig. 4). The small-bodied mountain-stream ancestor diverged from the main stem lineage 7.71 Mya (95% HPD 10.86–5.33 Mya). Divergence among the three Drakensberg Mountain species was initiated 3.95 Mya (95% HPD 5.95–2.32 Mya). Divergence within the Cape Fold Mountain lineage was initiated 5.90 Mya (95% HPD 8.40–3.95 Mya). Within the ancestral riverine lineage, divergence occurred 8.02 Mya (95% HPD 10.94–5.65 Mya), diverging into a temperate and tropical coastal forest lineage 5.29 Mya (95% HPD 7.60–3.39), with *P. valles*, being basal in the clade during the Late Miocene–Early Pliocene. The next divergence occurred when the ancestor radiated into subtropical and large-bodied riverine clades, 7.43 Mya (95% HPD 10.13–5.23 Mya). Within the subtropical clade, divergence was initiated 6.03 Mya (95% HPD 10.94–5.65 Mya) toward the Late Miocene. The latter clade contains most of the recently described diversity. In the large-bodied riverine species, divergence started 3.89 Mya (95% HPD 5.56–2.59 Mya), and the divergence between *P. karooensis* and *P. sidneyi s.s.* occurred 1.72 Mya (95% HPD 2.68–0.99 Mya) during the Plio/Pleistocene.

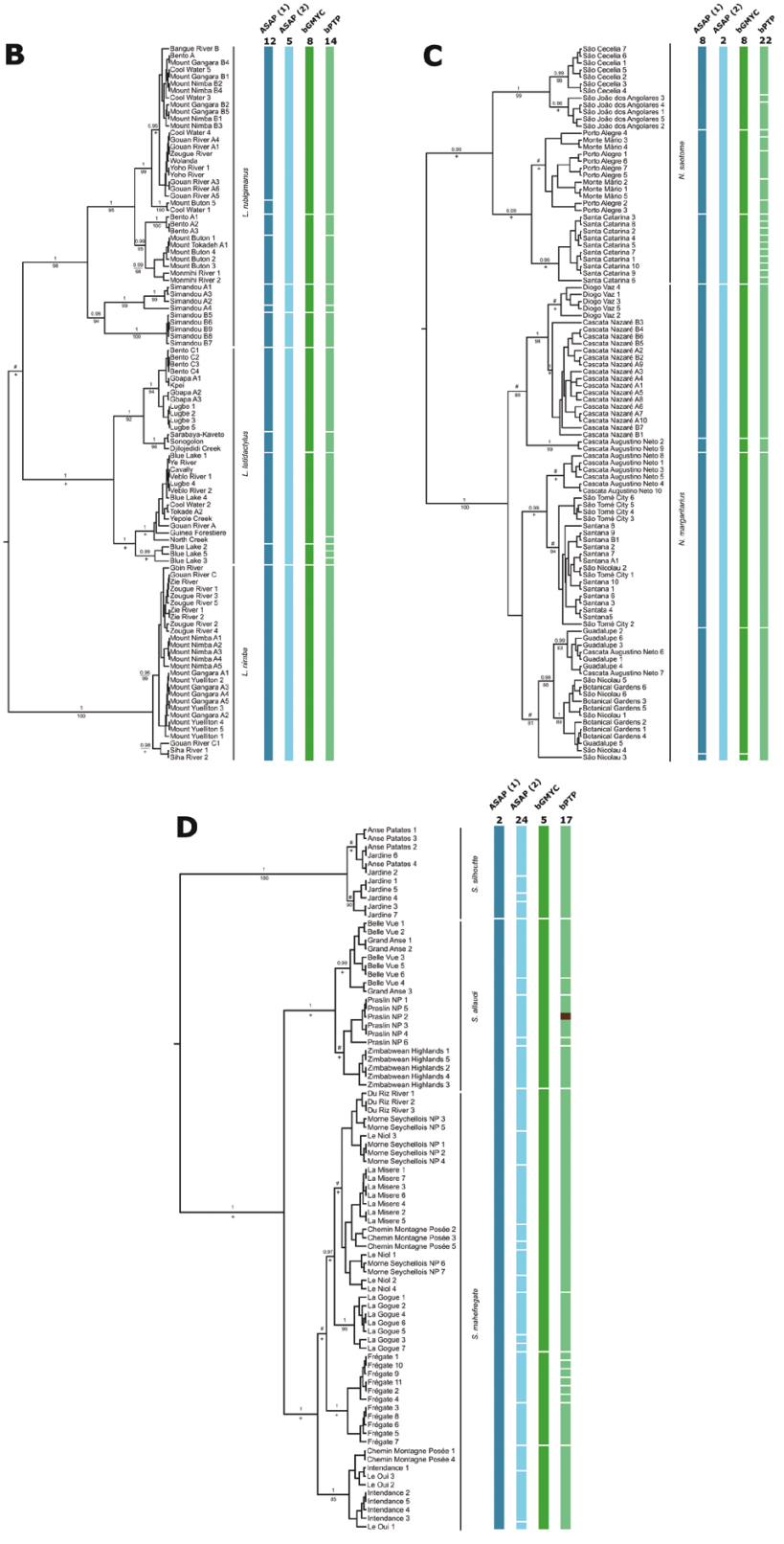

Figure 3. Continued.

Table 2. Four Afrotropical freshwater crab genera where *COI* sequence data were collected and subjected to three species delimitation methods (ASAP for the two partitions, bGYMC and bPTP). *N* represents the number of described species based on current taxonomy. Daniels *et al.* (2016) showed additional lineages in *Liberonautes* and suggest the presence of five species in their analyses

Dataset	Reference study	*COI* sequences	*N*	ASAP (1)	ASAP (2)	bGYMC	PTP
Seychellum	Daniels (2011)	83	3	2	24	5	17
Liberonautes	Daniels *et al.* (2016)	102	5	12	5	8	14
Nesonautes	Daniels & Klaus (2018)	102	2	8	2	2	22
Potamonautes	[+]Present study	323	20	15	18	28	53

[+]Reference studies for *Potamonautes* for which *COI* data were used: Gouws *et al.* (2015); Daniels *et al.* (2019), Daniels & Bayliss (2012); Phiri & Daniels (2014, 2016) and Wood & Daniels (2016).

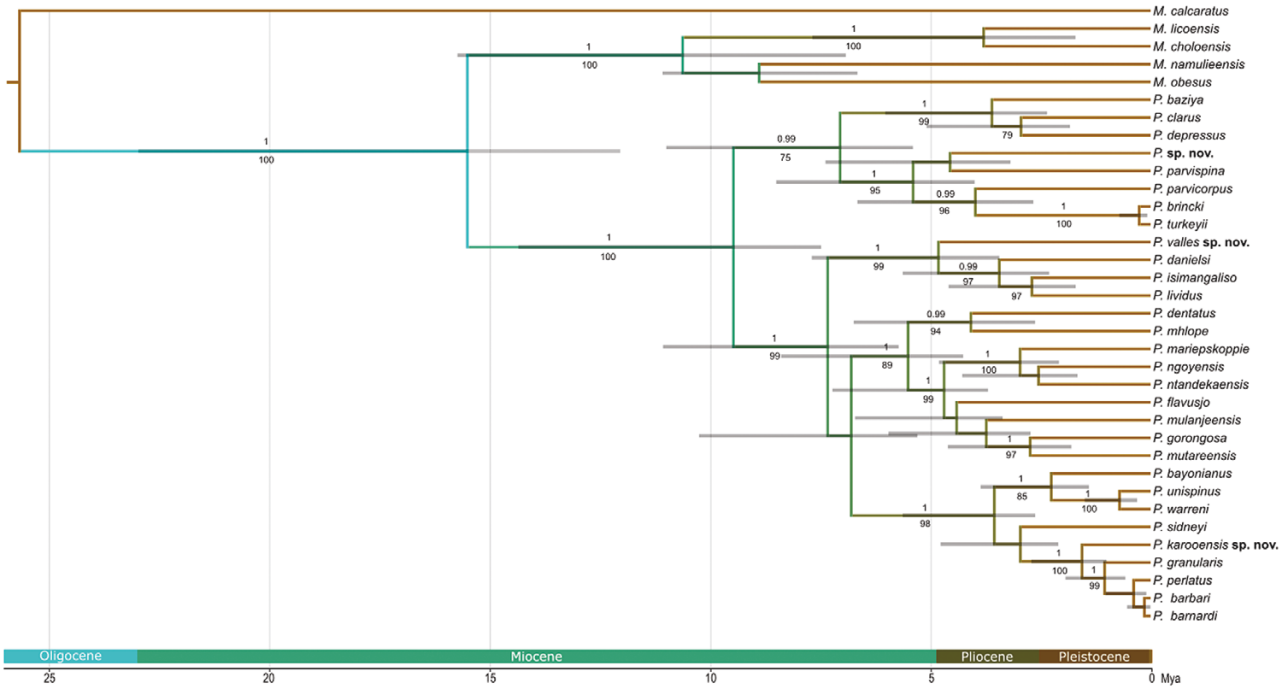

Figure 4. BEAST chronogram of the three concatenated mtDNA loci (16S rRNA, 12S rRNA + *COI*) dataset for all the described southern African *Potamonautes* species. Node bars show 95% highest posterior distributions for each divergence date estimate. The terminal labelled *P.* sp. nov., represents a yet undescribed species from Hogsback. Posterior probability values > 0.95 (PP) together with bootstrap values > 75% are shown above and below branches, respectively.

TAXONOMY

SUBORDER BRACHYURA LINNAEUS, 1758

SUPERFAMILY POTAMOIDEA ORTMANN, 1896

FAMILY POTAMONAUTIDAE BOTT, 1970

SUBFAMILY POTAMONAUTINAE BOTT, 1970

GENUS *POTAMONAUTES* MACLEAY, 1838

***POTAMONAUTES KAROOENSIS* SP. NOV.**

(FIGS 2, 4, 5 A–C, 6A, B, 7A–D; TABLE 3)

Zoobank registration: urn:lsid:zoobank.org:act:220C7F3B-1829-42AB-81C7-322F8464CBE2.

Holotype: Erasmuskloof farm, Eastern Cape Province, 32°16′ 44.0″ S, 24° 47′ 44.0″ E, 1326 m above sea level (a.s.l.) South Africa, SAM MB-A094477; one male. Collected by A. Barnes and S. R. Daniels, 2 May 2021 in a flowing stream (Fig. 3A, B).

Paratype: Asante Sana, Waterkloof hike, Eastern Cape Province, 32° 16′ 12.3″ S, 24° 57′ 25.4″ E, 1090 m a.s.l., South Africa, SAM MB-A094478; one male. Hand collected by A. Barnes and S. R. Daniels, 1 May 2021. Collected in a forested stream under rocks.

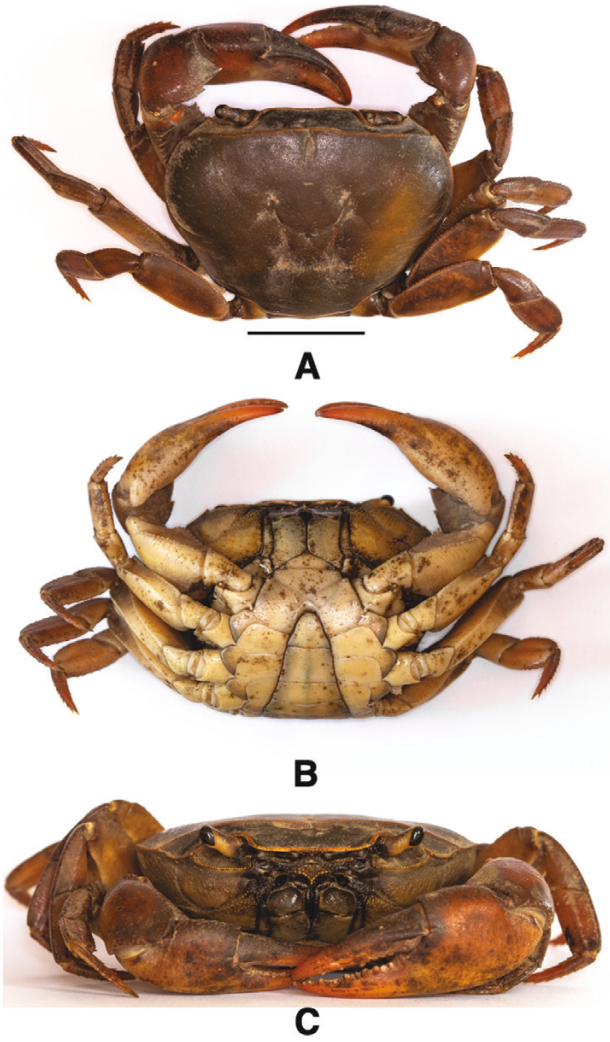

Figure 5. *Potamonautes karooensis* sp. nov., male holotype (CL = 47.48 mm) (SAM-MB A094477) Erasmuskloof, Eastern Cape Province, South Africa. A, whole animal dorsal aspect; B, whole animal ventral aspect; C, cephalothorax, frontal aspect. Scale bar represents 10 mm.

Additional material examined: Asante Sana, Waterkloof hike, 32° 16′ 12.3″ S, 24° 57′ 25.4″ E, 1090 m a.s.l., Eastern Cape Province, South Africa, SAM MB-A094479; four males and two females. Collected by A. Barnes and S. R. Daniels, 1 May 2021 in a flowing stream along the mountain side. Langfontein farm, 32° 11′ 8.12″ S, 24° 09′ 9.05″ E, 976m a.s.l., Eastern Cape Province, South Africa, SAM MB-A094480; four females. Collected by A. Barnes and S. R. Daniels, 2 May 2021. Hand collected in a flowing stream under boulders. Ouberg Pass, 32° 04′ 0.93″ S, 24° 21′ 4.20″ E, 1178 m a.s.l., Eastern Cape Province, South Africa, SAM MB-A094481, four females. Collected by A. Barnes and S. R. Daniels, 4 May 2021. Hand collected in stagnant water under a bridge.

Diagnosis: Carapace very flat (CH/CL = 0.43) (Table 3); postfrontal crest well-defined, complete, lateral ends meeting anterolateral margins; epigastric crests faint, median sulcus between crests short, not forked posteriorly; exorbital, epibranchial teeth reduced to granules; anterolateral carapace margin with small epibranchial tooth (Fig. 5A–C). Third maxilliped: ischium with distinct vertical sulcus; s3/s4 complete, V-shaped, deep, midpoint almost meeting anterior margin of sternopleonal cavity; margins of s4 low, not raised (Fig. 5B). Cheliped: dactylus (moveable finger) slim, highly arched, enclosing oval interspace, with three larger teeth interspersed by smaller teeth along length; propodus (fixed finger) with four larger teeth interspersed by smaller teeth along length (Fig. 6A, B); carpus inner margin distal tooth large, pointed, proximal tooth reduced to granules; medial inferior margin of merus lined with series of small granules terminating distally at small, low distal meral tooth, lateral inferior margin smooth. G1 terminal article: one-third length of subterminal segment; first-third straight in line with longitudinal axis of subterminal segment, middle part directed outward at 45°, widened by raised rounded ventral lobe, tip curving sharply upward (Fig. 6A, B).

Description: Based on male holotype (holotype CWW 63.95 mm, Table 3). Carapace with small distinct tooth on the anterolateral margins; widest anteriorly, narrowest posteriorly (CWP/CL 0.50); flattened (CH/CL 0.43) (Fig. 5C); front broad, one-third CWW (FW/CWW 0.35); urogastric, cardiac grooves distinct, other grooves faint or missing; postfrontal crest complete, anterolateral margin posterior to epibranchial tooth granulated, meeting epibranchial teeth; epigastric crests faint, median sulcus between crests short, forked posteriorly; exorbital, epibranchial teeth each reduced to granule; anterolateral margin between exorbital, epibranchial teeth faintly granulated, curving slightly outward, lacking intermediate tooth (Fig. 5A–C); branchiostegal wall vertical, sulcus faint, meeting longitudinal sulcus, dividing branchiostegal wall into three parts, suborbital, dorsal pterygostomial regions granulated, hepatic region smooth; suborbital margin faintly granulated. Third maxilliped: filling entire buccal frame, except for respiratory openings; exopod with long flagellum, ischium with faint vertical groove (Fig. 7D). Epistomial tooth large, triangular, margins lined by large granules. Mandible: palp two-segmented; terminal segment simple; tuft of setae at junction between segments. Sternum: s1, s2 fused; s2/s3 deep, completely crossing sternum; s3/s4 complete, V-shaped, deep, midpoint almost meeting anterior margin of

Table 3. *Potamonautes karooensis* sp. nov., measurements (in mm) of the holotype and the range of additional male and female specimens examined

Variable	Abbreviation	Holotype	Male	Females
carapace length	CL	47.48	46.08	48.20–37.43
carapace width at widest point	CWW	63.95	64.08	64.78–50.11
carapace posterior margin	CWP	23.75	20.80	23.35–17.58
frontal width	FW	23.01	24.34	27.01–17.96
distance between postfrontal crest and anterior margin	PFCD	5.43	6.29	6.55–4.32
carapace height	CH	20.85	22.43	25.37–17.15
major cheliped propodus length	MCPL	45.78	54.13	46.95–30.92
major cheliped dactylus length	MCDL	27.10	34.37	27.83–18.71
pereiopod 2, merus length	P2ML	23.56	24.90	22.33–18.54
pereiopod 2, merus width	P2MW	10.68	9.92	9.73–8.48
pereiopod 5, merus length	P5ML	22.17	23.90	24.05–18.89
pereiopod 5, merus width	P5MW	9.64	9.85	9.66–8.13

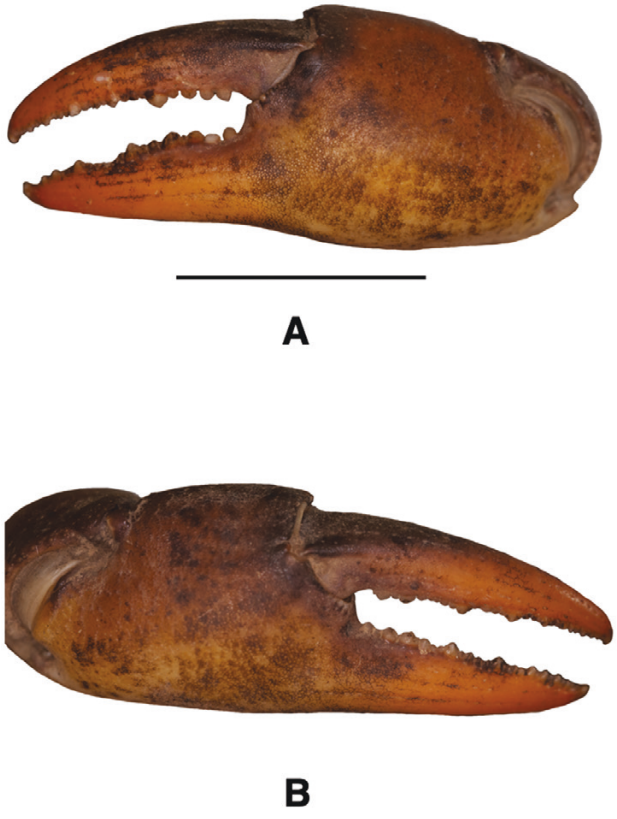

Figure 6. *Potamonautes karooensis* sp. nov., male holotype (SAM-MB A094477) A, major right cheliped; B, minor left cheliped. Scale bar represents 10 mm.

sternopleonal cavity; margins of s4 low, not raised. Cheliped: dactylus (moveable finger) slim, arched, enclosing oval interspace, with three larger teeth interspersed by smaller teeth along length; propodus (fixed finger) with four larger teeth interspersed by smaller teeth along length (Fig. 6A, B); carpus distal tooth large, pointed, proximal tooth small but distinct, followed by granule; both inferior margins of merus lined by series of small granules, distal meral tooth small, pointed. Pereopods: walking legs slender, pereopod 3 longest, pereopod 5 shortest; dorsal margins of pereopods with fine sharp bristles, dactyli of walking legs ending in sharp point, with rows of spine-like bristles along segment. Pleon: outline broadly triangular with straight margins. G1 terminal article: short (one-third length of subterminal segment), curving away from midline, first-third straight in line with longitudinal axis of subterminal segment, middle part directed outward at 45°, widened by low raised rounded ventral lobe, tip curving gently upward. G1 subterminal segment broad at base, tapering to slim junction with terminal article distally where these two parts have same width, ventral side of segment with heavily setose margins; with setae-fringed flap covering lateral half of segment; dorsal side of segment smooth, no flap, with broad membrane on the dorsal side of suture marking junction between terminal, subterminal parts (Fig. 7A, B). G2: terminal article long, flagellum-like, 0.5 times length of subterminal segment (Fig. 7C).

Molecular diagnosis: 16S rRNA GenBank accession numbers: OL685395–OL685398, OL685401–OL685411, OL685504. *COI* GenBank accession numbers: OL660552–OL660566, OL660588.

Distribution: Known from the Great Karoo Basin around the town of Graaff-Reinet, Eastern Cape Province, South Africa.

Remarks: Morphologically, *Potamonautes karooensis*, is most likely to be confused with *P. sidneyi*, *P. barbarai*, *P. barnardai*, *P. granularis* and *P. perlatus*. Geographically, *P. karooensis* is confined to the Great

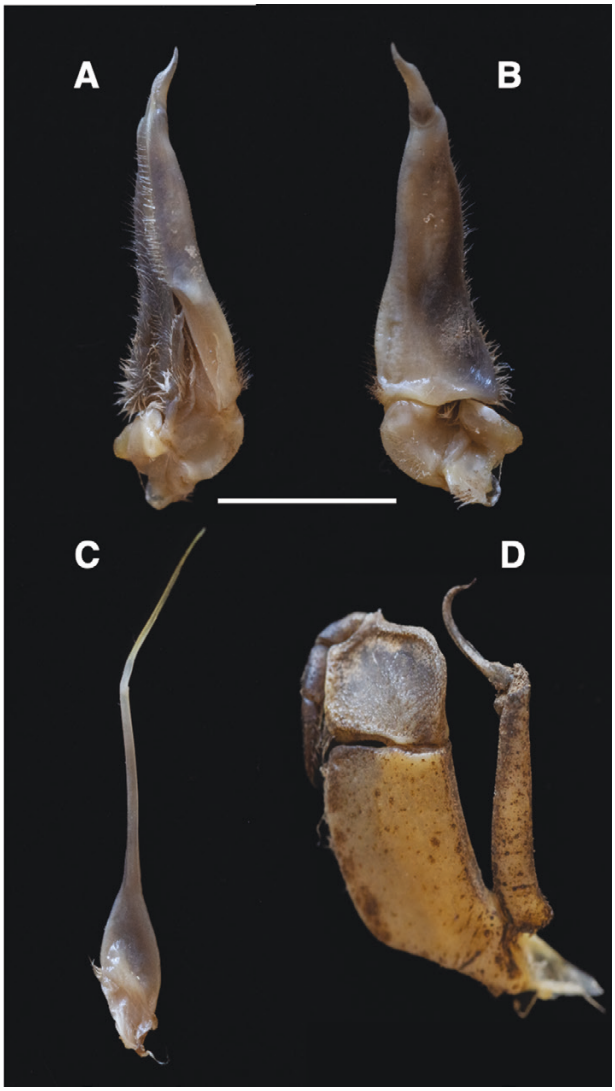

Figure 7. *Potamonautes karooensis* sp. nov., male holotype (SAM-MB A094477) A, left gonopod 1, anterior view; B, left gonopod 1 posterior view; C, left gonopod 2 anterior view; D, right third maxilliped. Scale bar represents 10 mm.

Karoo Basin, Eastern Cape Province, South Africa where it is present in small, temporary, high-lying mountain streams (Supporting Information, Fig. S3A). *Potamonautes karooensis*, is brown-coloured with orange-tipped chelipeds (Supporting Information, Fig. S3B). *Potamonautes perlatus* is also a large riverine species that is flat (CH/CL = 0.69) and wide (CWW = 60.5 mm) (Phiri & Daniels, 2014). The species occurs along the interior and coastal margins of the Western Cape Province where it is common in large rivers and streams at low altitude, such as the Berg, Bot, Eerste, Olifants and Doring rivers (Phiri & Daniels, 2014). *Potamonautes granularis* is present only in the lower Olifants River, Western Cape and is a large-bodied riverine species, with a granulated anterolateral carapace margins and orange-tipped chelipeds (Daniels *et al.*, 1998). In addition, two additional large-bodied riverine species are present in the Western Cape extending into the Eastern Cape provinces, South Africa. *Potamonautes barbarai* is present in the Gamtoos and Gourits river systems (in the Western and Eastern Cape provinces), the species is large (CWW = 63 mm) and has a swollen carapace (CH/CL = 0.58), while *P. barnardai* is present in the Breede River system, and is large-bodied (CWW = 54.6 mm) and flat (CH/CL = 0.61). *Potamonautes sidneyi s.s.* occurs in rivers, streams and wetlands in southern KwaZulu-Natal, Gauteng, Mpumalanga, Limpopo and the Northern Cape provinces of South Africa into Mozambique. The latter species is flat (CH/CL = 0.54) and wide (CWW = 52.4 mm) (Peer *et al.*, 2017). Geographically, the other large-bodied riverine species that *P. karooensis* occurs in close geographic proximity to is *P. danielsi*, despite being phylogenetically distantly related. *Potamonautes danielsi* is confined to the coastal forest rivers and streams in the Eastern Cape and southern KwaZulu-Natal provinces (Daniels *et al.*, 2022). *Potamonautes danielsi* is smaller-bodied (CWW = 25.8 mm) and flat (CH/CL = 0.49). Four described small-bodied, mountain-stream living crabs with highly arched dactyli are present in first and second order streams along the Cape Fold Mountains; these include *P. parvicorpus* (Cape Peninsula and Table Mountain, Jonkershoek, Helderberg Mountains), *P. parvispina* (Cederberg Mountains), *P. brincki* (Hottentots Holland Mountains), *P. tuerkayi* (Overberg Mountains), while an undescribed mountain-stream potamonautid crab is known from Hogback (N. Peer, pers. comm.).

Etymology: Named after the Great Karoo Basin of South Africa. A semi-desert region in the interior of South Africa.

POTAMONAUTES VALLES SP. NOV.

(FIGS 2, 4, 8A–C, 9A, B, 10A–D; TABLE 4)

Zoobank registration: urn:lsid:zoobank.org:act:6C7215BD-74DA-4780-9589-375930C5981C.

Holotype: Muilhuis section, Blyde River Canyon Nature Reserve, Northern Escarpment, 24° 40′ 14.8″ S, 30° 52′ 34.1″ E, 1320 m a.s.l., Mpumalanga Province, South Africa, SAM MB-A 094482, one male. Hand collected by H. Marais, 12 July 2021. Collected in fast-flowing mountain streams.

Paratype: Muilhuis section, Blyde River Canyon Nature Reserve, Northern Escarpment, 24° 40′ 14.8″ S, 30° 52′ 29.2″ E, 1308 m a.s.l., Mpumalanga Province, South Africa, SAM MB-A 094483, one male. Hand

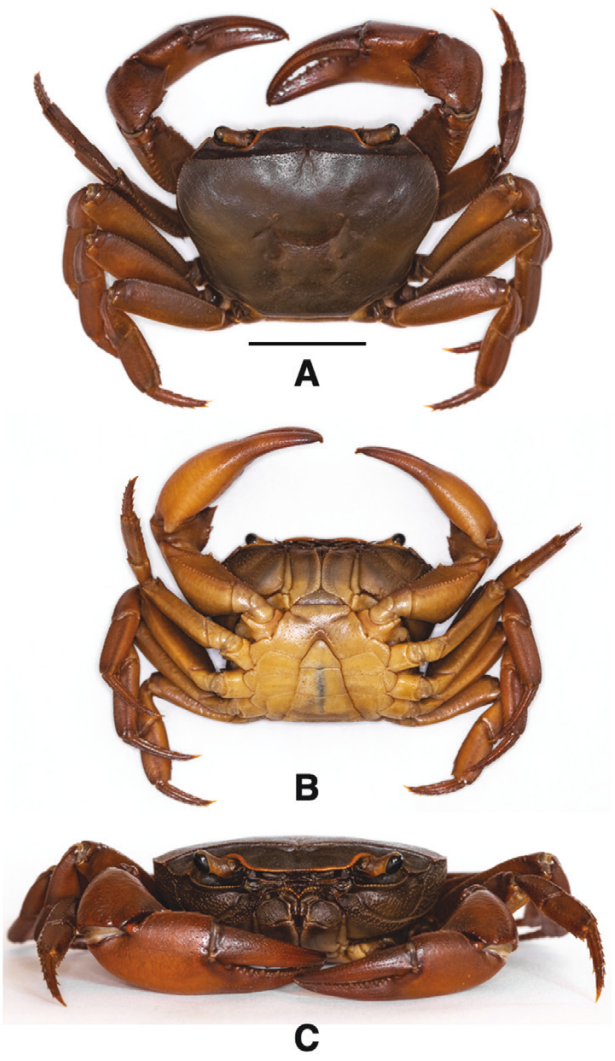

Figure 8. *Potamonautes valles* sp. nov., male holotype (CL = 30.90 mm) (SAM-MB A094482) from Muilhuis section, Blyde River Canyon Nature Reserve, Mpumalanga Province, South Africa. A, whole animal dorsal aspect; B, whole animal ventral aspect; C, cephalothorax, frontal aspect. Scale bar represents 10 mm.

collected by H. Marais, 12 July 2021. Collected in fast-flowing mountain streams.

Additional material examined: Muilhuis section, Blyde River Canyon Nature Reserve, Northern Escarpment, 24° 40′ 14.8″ S, 30° 52′ 34.1″ E, 1320 m a.s.l., Mpumalanga Province, South Africa, SAM MB-A 094484, one male. Collected by H. Marais, 25 November 2020. Collected in a fast-flowing mountain stream. Unnamed locality, 30 km north-east of Mbabane 26° 12′ 24.1″ S, 31° 17′ 59.6″ E, 1200 m a.s.l., Hhohho Province, Eswatini, 1 February 2019. One recently moulted male, SAM MB-A 094485 collected

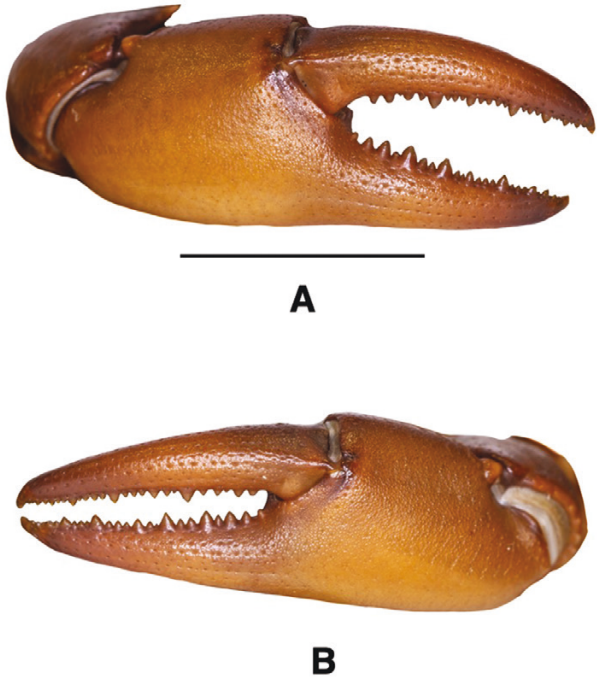

Figure 9. *Potamonautes valles* sp. nov., male holotype (SAM-MB A 094482). A, major right cheliped; B, minor left cheliped. Scale bar represents 10 mm.

by Theo Busschau. Unnamed locality, 30 km north-east of Mbabane 26° 12′ 35.4″ S, 31° 17′ 07.2″ E, 1200 m a.s.l., Hhohho Province, Eswatini, three males, five females, three juveniles, SAM MB-A 094486 collected 1 February 2019 by Theo Busschau. One adult male, SAM MB-A094580 from Katrinasrust trout farm, 25° 42′ 05″ S, 30° 30′ 38″ E, 1664 m, Mpumalanga Province, South Africa, collected 28 November 2021 by Graeme Gullacksen. One adult female, SAM MB-A094581 from Katrinasrust trout farm, 25° 42′ 05″ S, 30° 30′ 38″ E, 1664 m Mpumalanga Province, South Africa, collected 1 December 2021 by Graeme Gullacksen. One adult male, SAM MB-A094582 from Katrinasrust trout farm, 25° 42′ 05″ S, 30° 30′ 38″ E, 1664 m Mpumalanga Province, South Africa, collected 15 January 2022 by Graeme Gullacksen.

Diagnosis: Carapace very flat (CH/CL = 0.51) (Table 4); postfrontal crest well defined, complete, lateral ends meeting anterolateral margins; epigastric crests faint, median sulcus between crests short, not forked posteriorly; exorbital, epibranchial teeth reduced to granules; anterolateral carapace margin granulated (Fig. 8A–C). Third maxilliped: ischium with distinct vertical sulcus (Fig. 8C); s3/s4 complete, V-shaped, deep, midpoint almost meeting anterior margin of sternopleonal cavity; margins of s4 low, not raised (Fig. 8B). Cheliped: dactylus

Figure 10. *Potamonautes valles* sp. nov., male holotype (SAM-MB A 094482). A, left gonopod 1, anterior view; B, left gonopod 1 posterior view; C, left gonopod 2 anterior view; D, right third maxilliped. Scale bar represents 10 mm.

(moveable finger) slim, highly arched, enclosing oval interspace, with three larger teeth interspersed by smaller teeth along length; propodus (fixed finger) with four larger teeth interspersed by smaller teeth along length (Fig. 9A, B); carpus inner margin distal tooth large, pointed, proximal tooth reduced to granules (Fig. 8A); medial inferior margin of merus lined with series of small granules terminating distally at small, low distal meral tooth, lateral inferior margin smooth. G1 terminal article: one-third length of subterminal segment; first-third straight in line with longitudinal axis of subterminal segment, middle part directed outward at 45°, widened by raised rounded ventral lobe, tip curving sharply upward (Fig. 10A, B).

Description: Based on male holotype (holotype CWW 40.24 mm, Table 4). Carapace anterolateral margins granulated; widest anteriorly, narrowest posteriorly (CWP/CL 0.42); arched (CH/CL 0.51) (Fig. 8C); front broad, one-third CWW (FW/CWW 0.40); urogastric, cardiac grooves distinct, other grooves faint or missing; postfrontal crest complete, anterolateral margin posterior to epibranchial tooth granulated, meeting epibranchial teeth; epigastric crests faint, median sulcus between crests short, forked posteriorly; exorbital, epibranchial teeth each reduced to granule; anterolateral margin between exorbital, epibranchial teeth faintly granulated, curving slightly outward, lacking intermediate tooth (Fig. 8A–C); branchiostegal wall vertical sulcus faint, meeting longitudinal sulcus, dividing branchiostegal wall into three parts, suborbital, dorsal pterygostomial regions granulated, hepatic region smooth; suborbital margin faintly granulated. Third maxilliped: filling entire buccal frame, except for respiratory openings; exopod with long flagellum, ischium with faint vertical groove (Fig. 10D). Epistomial tooth large, triangular, margins lined by large granules. Mandible: palp two-segmented; terminal segment simple; tuft of setae at junction between segments. Sternum: s1, s2 fused; s2/s3 deep, completely crossing sternum; s3/s4 complete, V-shaped, deep, midpoint almost meeting anterior margin of sternopleonal cavity; margins of s4 low, not raised. Cheliped: dactylus (moveable finger) slim, arched, enclosing oval interspace, with three larger teeth interspersed by smaller teeth along length; propodus (fixed finger) with four larger teeth interspersed by smaller teeth along length (Fig. 9A, B); carpus distal tooth large, pointed, proximal tooth small but distinct, followed by granule; both inferior margins of merus lined by series of small granules, distal meral tooth small, pointed. Pereopods: walking legs slender, pereopod 3 longest, pereopod 5 shortest; dorsal margins of pereopods with fine sharp bristles, dactyli of walking legs ending in sharp point, with rows of spine-like bristles along segment. Pleon: outline broadly triangular with straight margins. G1 terminal article: short (one-third length of subterminal segment), curving away from midline, first-third straight in line with longitudinal axis of subterminal segment, middle part directed outward at 45°, widened by low raised rounded ventral lobe, tip curving gently upward. G1 subterminal segment broad at base, tapering to slim junction with terminal article distally where these two parts have same width, ventral side of segment with heavily setose margins; with setae-fringed flap covering lateral half of segment; dorsal side of segment smooth, no flap, with broad membrane on the dorsal

Table 4. *Potamonautes valles* sp. nov., measurements (in mm) of the holotype and the range of additional male and female specimens examined. An * indicates that the structure was missing

Variable	Abbreviation	Holotype	Males	Females
carapace length	CL	30.90	38.18–23.95	29.13–26.73
carapace width at widest point	CWW	40.24	50.91–31.07	38.92–34.82
carapace posterior margin	CWP	13.24	19.47–11.64	15.01–13.93
frontal width	FW	16.48	19.40–10.96	13.92–13.36
distance between postfrontal crest and anterior margin	PFCD	4.47	4.46–3.20	3.49–2.91
carapace height	CH	15.86	17.32–16.83	14.08–13.05
major cheliped propodus length	MCPL	31.38	40.22–18.90	23.91–21.09
major cheliped dactylus length	MCDL	19.22	23.75–10.78	13.86–12.93
pereiopod 2, merus length	P2ML	18.44	*–11.75	14.70–13.81
pereiopod 2, merus width	P2MW	6.53	*– 4.94	6.44–5.90
pereiopod 5, merus length	P5ML	17.76	*–12.77	15.91–14.11
pereiopod 5, merus width	P5MW	6.44	*–5.18	6.10–5.92

side of suture marking junction between terminal, subterminal parts (Fig. 10A, B). G2: terminal article long, flagellum-like, 0.5 times length of subterminal segment (Fig. 10C).

Molecular diagnosis: 16S rRNA GenBank accession numbers: OL685399–OL685400, OL685418–OL685422, OL685439–OL685441. *COI* GenBank numbers: OL660549–OL660551, OL660573–OL660577.

Distribution: Known from the Blyde River Canyon Nature Reserve, Katrinasrust trout farm (in the vicinity of Mbombela) and Sterkspruit in the north-east of the Mpumalanga Province in South Africa, as well as 30 km north-west of Mbabane, Eswatini (Fig. 1; Supporting Information, Fig. S4A, B).

Remarks: Potamonautes valles is sister to *P. danielsi* in our phylogenetic analyses (Fig. 4). Geographically the two species are distinct: *P. valles* is confined to the north-eastern corners of southern Africa, while *P. danielsi* occurs from southern KwaZulu-Natal along the Indian Ocean coastal (IOCB) forest belt and the adjacent interior into the Eastern Cape Province of South Africa. Morphologically the two can also be distinguished. *Potamonautes valles* is flat (CH/CL = 0.51) and large bodied (CWW = 40.24 mm), while *P. danielsi* is also flat (CH/CL = 0.49) but smaller bodied (CWW = 25.8 mm) (Peer *et al.*, 2017). The terminal segment of gonopod 1 is short and 0.21 times the length of subterminal segment (Peer *et al.*, 2017). *Potamonautes sidneyi s.s.*, is flat (CH/CL = 0.54) and large bodied (CWW = 47 mm). When alive, *P. valles* has a chocolate-brown colour with a purple pair of chelipeds with black tips (Supporting Information, Fig. S4C). In addition, two burrowing swamp forest dwelling species are in this clade, *P. isimangaliso* and *P. lividus*. *Potamonautes isimangaliso* is endemic to the False Bay region of the iSimangaliso Wetland Park in the north-eastern KwaZulu-Natal Province, while *P. lividus* is present along the IOCB forest belt in KwaZulu-Natal and the Eastern Cape provinces of South Africa (Daniels *et al.*, 2020b). Ecologically, both *P. isimangaliso* and *P. lividus* are distinct from *P. valles*, because these species burrow into soil in their respective habitats, while *P. valles* is a boulder-stream dwelling species. The cephalothorax of *P. lividus* is ovoid, the postfrontal crest is incomplete, the carapace is vaulted and the branchial regions are convex. *Potamonautes lividus* has a sharp but small exorbital tooth but lacks epibranchial teeth and the carapace is highly vaulted (CH/CL = 0.64), indicative of a semi-terrestrial mode of life (Gouws *et al.*, 2001). The species is moderately large (CWW = 37 mm) (Gouws *et al.*, 2001). The chelipeds are highly arched and adapted for burrowing (Gouws *et al.*, 2001). *Potamonautes lividus* has a silver-blue shine of its carapace and may also be red to dark orange in colour (Gouws *et al.*, 2001). The terminal segment of gonopod 1 is 0.25 times the length of the subterminal segment (Gouws *et al.*, 2001). Similarly, the cephalothrax in *P. isimangaliso* is also ovoid, lacks epibranchial teeth, the exorbital teeth are reduced, the carapace is highly vaulted (CH/CL = 0.57) and the species lacks any dentition on the anterolateral carapace margins (Peer *et al.*, 2015). *Potamonautes isimangaliso* varies in colour from light brown, maroon, purple or brown/black in colour while it is light orange between the joints (Peer *et al.*, 2015). *Potamonautes isimangaliso* is large bodied (CWW = 55.1 mm) and inhabits ephemeral pans in sand forest where it burrows into the soil to a depth of 2–50 cm (Peer *et al.*, 2015). The terminal segment of gonopod 1 is short and 0.23 times the length of the subterminal segment (Peer *et al.*, 2015). In the

Mpumalanga Province of South Africa, five additional species are present, *P. flavusjo*, *P. mariepskoppie*, *P. sidneyi s.s.*, *P. unispinus* and *M. calcaratus*. These species can be easily distinguished from *P. valles*. *Potamonautes flavusjo* is a semi-terrestrial, burrowing species that lives in marsh (vlei) areas adjacent to small streams. In *P. flavusjo* the habitat is burrows in peat soils adjacent to streams. The species burrows straight down into the peat soil to a depth of near 1 m, but the depth of the burrow will be determined by the water-table depth. During the winter months, the species seals the burrow entrance with soil from the tunnel and sits in a small, water-filled chamber at the base of the burrow. The species generally comes to the surface after the first summer rainfalls (Daniels, pers. obs.). The chelipeds of *P. flavusjo* show limited adaptations for burrowing (Daniels *et al.*, 2014). *Potamonautes flavusjo* is a large-bodied species (CWW = 58.42 mm) (Daniels *et al.*, 2014). The carapace of *P. flavusjo* is highly arched (CH/CL = 0.48) and the anterolateral margins of the carapace is smooth (Daniels *et al.*, 2014). In addition, *P. flavusjo* is sulphur yellow ventrally and has yellow spots on the dorsal carapace surface. The terminal segment of gonopod 1 is short and 0.24 times the length of the subterminal segment (Daniels *et al.*, 2014). *Potamonautes mariepskoppie* is a narrow endemic species confined to the Mariepskop area of the Blyde River Canyon Nature Reserve where it occurs in swampy areas. The species has a modified cheliped, and the dactylus is large and shovel-shaped and adapted for burrowing into soft mud (Daniels *et al.*, 2021). Unpublished genetic data corroborate the presence of the species at Haenertsburg in the Limpopo Province (Daniels, unpublished). In *P. valles*, the dactylus and propodus are both highly arched, a pattern typically observed in stream-dwelling mountain crab species that live under rocks and boulders, and are not associated with a burrowing mode of life. In addition, in *P. valles*, the anterolateral carapace margin lacks any dentition. *Potamonautes unispinus* has a single tooth on the anterolateral carapace margin and is common and widespread in rivers (Stewart & Cook, 1997). *Potamonautes valles* can be differentiated from *P. sidneyi s.s.* by its highly arched dactylus and propodus, and its confinement to fast-flowing mountain streams. *Maritimonautes calcaratus* is phylogenetically distinct from *P. valles*, with a near ovoid and arched carapace (CH/CL = 0.51), and a thin spine-like tooth on the anterolateral carapace margin (Reed & Cumberlidge, 2004; Cumberlidge & Daniels, 2022). In *P. valles*, the anterolateral carapace margin is granulated and it lacks a spine. The chelipeds (both propodus and dactylus) of *M. calcaratus* are flat and broad and adapted for digging (Daniels, pers. obs). *Maritomonautes calcaratus* inhabits ephemeral pans, where it burrows into the soft, sandy edges of the pans to a depth of 70 cm (Daniels *et al.*, 2002). The terminal article of gonopod 1 in *M. calcaratus* is short in comparison with *P. valles* (Cumberlidge & Daniels, 2022). The distribution of *M. calcaratus* in South Africa is restricted to the Mpumalanga Province where it is present in the Kruger National Park, and the species is also present in neighbouring Mozambique (Reed & Cumberlidge, 2004; Cumberlidge & Daniels, 2008). The distribution range and habitat of *M. calcaratus* does not overlap with *P. valles*.

Etymology: Named for its presence in valleys, hence the Latin word *valles* was used as a species epithet. It is a noun in apposition.

DISCUSSION

The three species delimitations (ASAP, bGYMC and bPTP) retrieved varying, albeit limited degrees of success in delineating species among the four Afrotropical freshwater crab genera based on the *COI* datasets (Table 2; Fig. 3A–D). Among the three methods, the ASAP (partition two) analyses retrieved the highest degree (50%) of congruence with the number of described species based on the existing morphological taxonomy for two freshwater crab genera (*Liberonautes* and *Nesonautes*). Within *Potamonautes*, *P. karooensis*, was recovered as distinct by ASAP (partitions one and two), while *P. valles* was supported as a distinct lineage by all three species delimitation methods. In addition, five *Potamonautes* species were consistently retrieved by all three delimitation methods (Fig. 3A–D). The distance-based ASAP is thought to perform well in the presence of low rates of speciation (Puillandre *et al.*, 2021). We observed similar results in *Nesonautes*, where ASAP (partition two) and bGYMC corroborated the presence of the two morphologically described species. In contrast, bPTP exhibited limited congruence with the number of species defined based on morphology and overestimated the number of MOTUs. Several studies have observed over- or underestimations in the possible number of MOTUs using these two coalescent-based methods (Zhang *et al.*, 2013; García-Melo *et al.*, 2019; Ellepola *et al.*, 2021). Both bGMYC and bPTP consider the evolutionary relationship of sequences, while bGMYC attempts to maximize the likelihood score of an ultrametric tree for intra- and interspecific processes; bPTP attempts to determine the transition point from a between to a within species process (Kapli *et al.*, 2017). Considering that bPTP uses substitutions directly, it does not require an ultrametric tree topology or a sequence input threshold, and it frequently yields better results compared to bGYMC. We did not observe these results in our study. However, bPTP does not

account for highly divergent intraspecific variation. In our study, where marked intraspecific variation and genetic differentiation was observed among species within all four Afrotropical genera, bPTP overestimated the number of MOTUs. Both bGMYC and bPTP are known to perform poorly when the contrast of intra- versus interspecific divergence is low, which was especially evident for clades 3 and 5. The latter can further be influenced by mutation rates, population sizes and the degree of within-species population structure, all of which could have played a role in the incongruency observed in the present study (Pante *et al.*, 2015; Ritchie *et al.*, 2018; Pentinsaari *et al.*, 2017). Barley *et al.* (2018) cautioned against the exclusive reliance of species delimitation methods in taxonomic studies and argued that, at present, we have a limited understanding of the complexities of these methods (with respect to model sensitivity, coalescent parameters, gene flow, population structure, in-species complexes and speciation rates). Future species delimitation methods should also incorporate the geographic aspects of the species distribution and morphological character sets, since these may aid species recognition. Furthermore, incongruence between the three species delimitation methods has been attributed to the sensitivity of the analyses to detect structure and violations in the assumptions of these analyses. For example, coalescent-based methods are dependent on the input tree and selected priors. Moreover, we are cognizant that we used only *COI* data, a rapidly evolving, maternally inherited locus that reflects only a single the gene tree. The use of a single locus can bias results, particularly where the marker is saturated (not present in our data).

Sensitive nuclear DNA markers, capable of resolving fine-scale differences, need to be incorporated into systematic studies. Nuclear DNA sequence loci for decapods are notoriously slowly evolving and since most of the divergence we observed in the present study is relatively recent, these markers would be of limited utility in recognizing these species. Phiri & Daniels (2016) reported that in the Drakensberg Mountain crabs, the two nuclear sequence markers employed were near invariant and unsuitable for phylogeographic inference. In addition, Daniels & Klaus (2018) reported genetic invariance between two mtDNA clades of *N. margaritarius* (A.Milne-Edwards, 1869) using the histone 3 (*H3*) locus from the Island of São Tomé. The genetic invariance of widely used nuDNA sequence markers among recently diverged groups of decapods remains problematic; therefore, rapidly evolving genomic DNA markers are urgently required to aid taxonomic resolution. Recent comparative studies using first and next generation sequencing platforms in Eumetazoa have been complementary and revealed high levels of congruence with respect to the number of MOTUs recovered by species delimitation methods (Ramírez-Portilla *et al.*, 2022). While our results are derived from three mtDNA loci, nuclear genetic data that differentiates *P. sidneyi s.s.* and *P. valles*, is available (Fig. 2; Supporting Information, Fig. S1). An allozyme study by Stewart & Cook (1998) reported the presence of a near fixed allele difference at the peptidase glycyl leucine (*GL*) locus between Sterkspruit (*P. valles*) and *P. sidneyi s.s.* from Vaal, Wakkerstroom, Mukhasa and Tchiombedi (Supporting Information, Fig. S1), while dramatic allele frequency shifts were observed for glucose phosphate isomerase (*GPI*) and phosphoglucomutase (*PGM*).

Our phylogenetic results demonstrated the presence of two novel lineages nested within the *Potamonautes sidneyi s.l.* species complex (Fig. 2). Clade 2 was comprised exclusively of specimens from the interior mountainous areas of the Great Karoo Basin in the, Eastern Cape Province, while clade 4 (Fig. 2) was exclusive to the highlands in the southern Mpumalanga Province of South Africa and neighbouring Eswatini. When representatives of clades 2 were placed in a phylogenetic context (Fig. 4), they were shown to be equidistant between *P. sidneyi s.s.* and *P. perlatus*, while specimens from clade 4 were sister to *P. danielsi*. If we consider both clades as conspecific it will render *P. sidneyi s.l.* paraphyletic. Species paraphyly can be attributed to several factors, among these the most prominent are poor taxonomy, introgression (intraspecific hybridization) and incomplete lineages sorting (Funk & Omland, 2003). The failure of alleles when recently diverged may also result in non-monophyletic species, particularly in recently diverged groups. In the latter instances, monophyly as a basis for species identification could be troublesome. While it is difficult to differentiate between the three competing hypotheses for paraphyly, given our exclusive mtDNA dataset, we favour the hypothesis that our results reflect inaccurate taxonomic designation.

Cladogenesis in the southern African *Potamonautes* was initiated during the Early to Late Miocene and continued into the Plio/Pleistocene. The divergence of the Greater Karoo and Mpumalanga/Eswatini clades can be associated with Miocene/Pliocene/Pleistocene climatic changes in the region (Fig. 4). The Late Miocene was globally characterized by cooling and enhanced aridification. Consequently, the habitat of aquatic taxa would have contracted with xeric habitats becoming more widespread. This trend continued into the Plio/Pleistocene. The Late Pliocene was characterized by an intensification of aridification. This period in subcontinental southern Africa is thought to have seen the continuation and enhancement of xeric/mesic cycles enhancing habitat fragmentation, resulting in a general decrease in precipitation and increased temperatures. These climatic factors likely contracted

the inland aquatic habitat to higher-lying regions, limiting gene flow among lower-lying population and promoting cladogenesis. Shi *et al.* (2021) demonstrated that precipitation and temperatures were important abiotic drivers of divergence in a Chinese freshwater crab species complex, *Sinopotamon yangtsekiense* Bott, 1967. It is evident from our results that episodes of aridification resulted in major habit shifts, which probably restricted aquatic fauna to refugial areas promoting divergence. Furthermore, the Miocene/Pliocene/Pleistocene represents epochs of major cladogenic activity among several endemic terrestrial faunal lineages in South Africa (Engelbrecht *et al.*, 2013; Diedericks & Daniels, 2014; Hofmeyr *et al.*, 2017; Busschau *et al.*, 2019, 2021; Taylor *et al.*, 2020; Raphalo *et al.*, 2021b).

Why the discrepancy between the molecular species delimitations and the morphologically based alpha-taxonomy of the four freshwater crab genera? The morphological designations may potentially be an underestimation based on the traditional taxonomic characters, such as gonopods 1 and 2, and carapace morphology, which may be highly conserved at times. However, we are convinced that all three species delimitation methods overestimated the diversity within the four genera. While the ASAP analyses provided a more conservative estimation of the number of species, the two remaining methods (bGYMC and bPTP) frequently overestimated the taxonomic diversity in the presence of genetically highly structured species where several clades were present. Hence, we interpret the taxonomic results of the present study with caution. Much of the novel diversity in South Africa and elsewhere in the Afrotropical region are being delineated with the aid of molecular studies (Daniels, 2011, Daniels *et al.*, 2014; Phiri & Daniels, 2016; Peer *et al.*, 2017), since many of the traditional morphological characters such as gonopods 1 and 2, carapace and mandibular palp features exhibit only subtle different between cryptic lineages. For example, using genetic data Cumberlidge *et al.* (2021) revealed the presence of two new species within the geographically widespread species *Lirrangopotamonautes lirrangensis* (Rathbun, 1904). Similarly, within the monophyletic Malagasy freshwater crab fauna a taxonomic revision by Cumberlidge *et al.* (2020) demonstrated the presence of five new genera and several new species based on mtDNA sequence data. In our study, we found marginal differences in gonopods 1 and 2 morphology among sister-freshwater crab species. A recent molecular systematic study of the Neotropical freshwater crab genus *Fredius* Pretzmann, 1967 also reported discordance between genetic clades and species based on gonopod 1 morphology (Mantelatto *et al.*, 2022).

The uncorrected '*p*' *COI* sequence distances between sister-species were generally > 7%, including the two novel taxa (clades 2 and 4, Fig. 2) and their sisters. For example, a value of 7.90% was reported between *P. lividus* and *P. isimangaliso*, and a value of 8.12% was reported between *P. dentatus* and *P. mhlophe* and, finally, a value of 8.10% was reported between *P. ntendekaensis* and *P. ngoyensis* (Wood & Daniels, 2016; Daniels, 2017; Daniels *et al.*, 2019). Similarly, an uncorrected '*p*'-distance of 4.92% was recorded between *N. margaritarius* and its sympatric sister *N. soatomensis* Cumberlidge & Daniels, 2018 (Daniels & Klaus, 2018). Phiri & Daniels, (2016) reported a mean *COI* distance value of 9.81% in the *P. clarus*/*P. depressus* species complex. The values we recorded in the present study compare favourably with those from earlier systematic studies (Gouws *et al.*, 2015; Daniels, 2017; Daniels & Klaus, 2018). Notably, uncorrected '*p*' *COI* distance values may be variable and should be interpreted cautiously when recognizing novel lineages, and these values should be integrated into a framework with additional character suites, such as, morphological, ecological and phylogenetic data to validate the designation of novel taxa.

We used the following six, more conservative criteria in an integrative framework to recognize putative lineages: (1) clades (by implication monophyletic) that were statistically well supported; (2) geographic exclusivity of clades; (3) subtle morphological differences between sister-species in gonopods 1 and 2; (4) marked, uncorrected '*p*' *COI* sequence distance values > 7%; and (5) the overall paraphyly observed when we included the representative DNA sequence data from clades 2 and 4 into the phylogenetic framework for the southern African freshwater crabs. Here, *P. karooensis* was equidistant to *P. sidneyi s.s.* and *P. perlatus*, while *P. valles* was sister to *P. danielsi* and, in the latter instance, also geographically distinct (Fig. 4). Finally, as criterion (6), we consider congruence among the three species delimitation methods in supporting clades 2 and 4 as distinct. We define a species as an irreducible group whose members decent from a common ancestor and possess a combination of certain fixed character differences equating to a phylogenetics species concept (Nixon & Wheeler, 1990). We are convinced that our results provide overwhelming evidence for the presence of two novel river crab species. We advocate that taxonomists (where possible) employ both molecular and morphological characters to delineate species boundaries and to explore the congruence among character sets. Species delimitation methods, notwithstanding their limitations, provide a useful guide tool to explore the possible presence of novel lineages.

ACKNOWLEDGEMENTS

We thank the permit authorities in the Eastern Cape and KwaZulu-Natal for issuing collection permits. Staff of KZN Wildlife and Miss R. van Reenen are thanked for assisting in the field with sample collection. We also thank the DNA sequencing facility at Stellenbosch University and the Department of Botany and Zoology for logistical support. Mr H. Barnard is thanked for collecting the sample from Papkuilsfontein. The South African Museum is thanked for issuing accession numbers for the freshwater crab specimens. All the wonderful and friendly farmers in the Karoo are thanked for allowing us to sample on their respective farms. Two anonymous reviewers are thanked for critical comments that improved the quality of the manuscript.

DATA AVAILABILITY

The 16S rRNA data are available at GenBank (accession numbers for *P. karooensis*, OL685395–OL685398, OL685401–OL685411; *P. sidneyi s.s.*, OL685412–OL685417, OL685423–OL685238, OL685442–OL68503; *P. danielsi*, OL685356–OL685368; *P. valles*, OL685399–OL685400, OL685418–OL685422, OL685439–OL685440; *P. barbarai*, OL685504). The *COI* data are available at GenBank (accession numbers OL660567–OL660572, OL660578–OL660587 for *P. sidneyi s.s.*; OL685173–OL685185 for *P. danielsi*; OL660552–OL660566 for *P. karooensis* for *P. valles*, OL660549–OL660551, OL660573–OL660577; for *P. barbarai* OL660588).

REFERENCES

Akaike H. 1973. Information theory as an extension of maximum likelihood principle. In: Petrov BN, Csake F, eds. *Second International Symposium on Information Theory*. Budapest: Akademiai Kaido, 267–281.

Barley AJ, **Brown JM**, **Thomson RC. 2018.** Impact of model violations on the inference of species boundaries under the multispecies coalescent. *Systematic Biology* **67:** 269–284.

Barnard KH. 1950. Descriptive catalogue of South African decapod Crustacea (crabs and shrimps). *Annals of the South African Museum* **38:** 1–837.

Barnes A, **Reiss T**, **Daniels SR. 2020.** Systematics of the *Peripatopsis clavigera* species complex (Onychophora: Peripatopsidae) reveals cryptic cladogenic patterning, with the description of five new species. *Invertebrate Systematics* **34:** 569–590.

Bott R. 1955. Die Süßwasserkrabben von Africa und ihre Stammesgeschichte. *Annales du Musée Royal du Congo Belge série* **3:** 209–352.

Bott R. 1960. Crustacea (Decapoda): Potamonidae. In: Hanström B, Brinck P, Rudebeck G, eds. *South African animal life: results of the Lund University Expedition in 1950–1952*, Vol. 7. Stockholm: Almqvist & Wiksell, 13–18.

Bott R. 1970. Betrachtungen über die Entwicklungsgeschichte und Verbreitung der Süßwasser Krabben nach der Sammlung des Naturhistorischen Museums in Genf/Schweiz. *Revue Suisse de Zoologie* **77:** 327–344.

Brito-Capello Fde. 1864. Descripção de tres especies novas de crustaceos da Africa occidental e observaçoÞes aìcerca do Penaeus Bocagei Johnson especie nova dos mares de Portugal. *Memorias da Academia Real das Sciencias de Lisboa* **3:** 1–11.

Busschau T, **Conradie W**, **Daniels SR. 2019.** Evidence for cryptic diversification in a rupicolous forest-dwelling gecko (Gekkonidae: *Afroedura pondolia*) from a biodiversity hotspot. *Molecular Phylogenetics and Evolution* **139:** 106549.

Busschau T, **Conradie W**, **Daniels SR. 2021.** One species hide many: molecular and morphological evidence for cryptic speciation in a thread snake (Leptotyphlopidae: *Leptotypholps sylvicolus* Broadley & Wallach, 1997). *Journal of Zoological Systematics and Evolutionary Research* **59:** 195–221.

Calman WJ. 1918. A new river crab from the Transvaal. *Annals of the Magazine of Natural History* **9:** 234–236.

Chace FA. 1953. Zoological results of a fifth expedition to east Africa. *Bulletin of the Museum of Comparative Zoology* **110:** 425–443.

Clement M, **Posada D**, **Crandall KA. 2000.** TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9:** 1657–1659.

Cumberlidge N, **Daniels SR. 2008.** A conservation assessment of the freshwater crabs of southern Africa (Brachyura: Potamonautidae). *African Journal of Ecology* **46:** 74–79.

Cumberlidge N, **Daniels SR. 2014.** Recognition of two new species of freshwater crabs from the Seychelles based on molecular evidence (Potamoidea: Potamonautidae). *Invertebrate Systematics* **28:** 17–31.

Cumberlidge N, **Daniels SR. 2018.** A new species of *Potamonautes* from São Tomé Island, Central Africa, with redescriptions of *P. margaritarius* (A. Milne-Edwards, 1869) from São Tomé and *P. principe* Cumberlidge, Clark, and Baillie, 2002, from Príncipe (Decapoda: Potamonautidae). *Contributions to Zoology* **87:** 287–303.

Cumberlidge N, **Daniels SR. 2022.** A new multilocus phylogeny reveals overlooksed diversity in African freshwater crabs (Brachyura: Potamoidea): a major revision with new higher taxa and genera. *Zoological Journal of the Linnean Society* **194:** 1268–1311.

Cumberlidge N, **Sachs R. 1989.** Three new subspecies of the West African freshwater crab *Liberonautes latidactylus* (de Man, 1903) from Liberia, with notes on their ecology. *Zeitschrift für Angewandte Zoologie* **76:** 425–439.

Cumberlidge N, **Soma JB**, **Leever EM**, **Daniels SR. 2020.** New genera within the Malagasy freshwater crab fauna: *Agora* n. gen., for *Thelphusa goudoti* H. Milne Edwards, 1853, and a phytotelmic new species of *Malagasya* Cumberlidge and Sternberg, 2002 (Brachyura: Potamonautidae: Deckeniidae). *Journal of Crustacean Biology* **40:** 584–599.

Cumberlidge N, **Johnson E**, **Clark P**, **Genner MJ. 2021.** Unravelling the *Potamonautes lirrangensis* (Rathbun, 1904) species complex (Potamoidea: Potamonautidae) with the description of two new species. *European Journal of Taxonomy* **744:** 145–178.

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

Daniels SR. 2011. Reconstructing the colonisation and diversification history of the endemic freshwater crab (*Seychellum alluaudi*) in thegranitic and volcanic Seychelles Archipelago. *Molecular Phylogenetics and Evolution* **61:** 534–542.

Daniels SR. 2017. Sympatric colour morphs or distinct species? Examining species boundaries amongst two South African freshwater crabs (Decapoda: Potamonautidae: *Potamonautes* MacLeay, 1838) with the description of a new species. *Journal of Crustacean Biology* **37:** 723–731.

Daniels SR, **Bayliss J. 2012.** Neglected refugia of biodiversity: mountainous regions in Mozambique and Malawi yield two novel freshwater crab species (Potamonautidae: *Potamonautes*). *Zoological Journal of the Linnean Society* **164:** 498–509.

Daniels SR, **Klaus S. 2018.** Divergent evolutionary origins and biogeographic histories of two freshwater crabs (Brachyura: *Potamonautes*) on the West African conveyer belt islands of São Tomé and Príncipe. *Molecular Phylogenetics and Evolution* **127:** 119–128.

Daniels SR, **Stewart BA**, **Gibbons M. 1998.** *Potamonautes granularis* sp. nov., (Brachyura, Potamonautidae) a new cryptic species of river crab from the Olifants River system, South Africa. *Crustaceana* **71:** 885–903.

Daniels SR, **Stewart BA**, **Cook PA. 2002.** Congruent patterns of genetic variation in a burrowing freshwater crab revealed by allozyme and mtDNA analysis. *Hydrobiologia* **468:** 171–179.

Daniels SR, **Gouws G**, **Crandall KA. 2006.** Phylogeographic patterning in a freshwater crab species (Decapoda: Potamonautidae: Potamonautes) reveals the signature of historic climatic oscillations. *Journal of Biogeography* **33:** 1538–1549.

Daniels SR, **Phiri EE**, **Bayliss J. 2014.** Renewed sampling of inland aquatic habitats in southern Africa yields two novel freshwater crab species (Decapoda: Potamonautidae: *Potamonautes*). *Zoological Journal of the Linnean Society* **171:** 356–369.

Daniels SR, **Phiri EE**, **Klaus S**, **Albrecht C**, **Cumberlidge N. 2015.** Multilocus phylogeny of the Afrotropical freshwater crab fauna reveals historical drainage connectivity and transoceanic dispersal since the Eocene. *Systematic Biology* **64:** 549–567.

Daniels SR, **Dambire C**, **Klaus S**, **Sharma PP. 2016.** Unmasking alpha diversity, cladogenesis and biogeographical patterning in an ancient panarthropod lineage (Onychophora: Peripatopsidae: *Opisthopatus cinctipes*) with the description of five novel species. *Cladistics* **32:** 506–537.

Daniels SR, **Busschau T**, **Cumberlidge N. 2019.** Two new species of freshwater crabs of the genus *Potamonautes* MacLeay, 1838 (Decapoda: Brachyura: Potamonautidae) from the forests of KwaZulu-Natal, South Africa. *Journal of Crustacean Biology* **39:** 426–435.

Daniels SR, **Bittencourt-Silva GB**, **Muianga V**, **Bayliss J. 2020a.** Phylogenetics of the freshwater crab (*Potamonautes* MacLeay, 1838) fauna from 'sky islands' in Mozambique with the description of a new species. *European Journal of Taxonomy* **716:** 1–23.

Daniels SR, **James N**, **Gouws G. 2020b.** Phylogeographic structure and continued surveys of a vulnerable South African freshwater crab (Potamonautidae, *Potamonautes lividus*): implications for the IUCN Red Listing of the Afrotropical fauna. *Aquatic Conservation of Marine and Freshwater Ecosystems* **30:** 2221–2239.

Daniels SR, **Barnes A**, **Marais H**, **Gouws G. 2021.** Surveys of Afrotemperate forests yields two new freshwater crabs (Decapoda: Potamonautidae: *Potamonautes*) from South Africa. *European Journal of Taxonomy* **782:** 82–107.

Diedericks G, **Daniels SR. 2014.** Ain't no mountain high enough, ain't no valley low enough? Phylogeography of the rupicolous Cape gridle lizard (*Cordylus cordylus*) reveals a generalist pattern. *Molecular Phylogenetics and Evolution* **71:** 234–248.

Drummond AJ, **Ho SY**, **Phillips MJ**, **Rambaut A. 2006.** Relaxed phylogenetics and dating with confidence. *PLoS Biology* **4:** e88.

Drummond AJ, **Rambaut A. 2007.** BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7:** 214.

Ellepola G, **Herath J**, **Manamendra-Arachchi K**, **Wijayathilaka N**, **Senevirathne G**, **Pethiyagoda R**, **Meegaskumbura M. 2021.** Molecular species delimitation of shrub frogs of the genus *Pseudophilautus* (Anura, Rhacophoridae). *PLoS One* **16:** e0258594.

Engelbrecht HM, **van Niekerk A**, **Heideman NJL**, **Daniels SR. 2013.** Tracking the impact of Pliocene/Pleistocene sea level and climatic oscillations on the cladogenesis of the Cape legless skink, *Acontias meleagris* species complex. *Journal of Biogeography* **40:** 492–506.

Funk DJ, **Omland KE. 2003.** Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution and Systematics* **34:** 397–423.

García-Melo JE, **Oliveira C**, **Da Costa Silva GJ**, **Ochae-Orrego E**, **Pereira LHG**, **Maldonado-Ocampo JA. 2019.** Species delimitation of neotropical characins (Stevardiinae): implications for taxonomy of complex groups. *PLoS One* **14:** e0216786.

Gordon I. 1929. A new river-crab of the subgenus *Potamonautes* from Portuguese East Africa. *Annals of the Magazine of Natural History* **3:** 405–411.

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

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

Gouws G, **Peer N**, **Perissinatto R. 2015.** mtDNA lineage diversity of a potamonautid freshwater crab in KwaZulu-Natal, South Africa. *Koedoe* **57:** 1–12.

Heled J, **Drummond AJ. 2010.** Bayesian inference of species trees from multilocus data. *Molecular Biology and Evolution* **27:** 570–580.

Hofmeyr MD, **Vamberger M**, **Branch W**, **Schleicher A**, **Daniels SR. 2017.** Tortoise (Reptilia, Testudinidae) radiations in southern Africa from the Eocene to the present. *Zoologica Scripta* **46:** 380–400.

Jacobs SJ, **Kristofferson C**, **Uribe-Convers S**, **Latvis M**, **Tank DC. 2018.** Incongruence in molecular species delimitation schemes: what to do when adding more data is difficult. *Molecular Ecology* **27:** 2397–2413.

Jesse R, **Schubart CD**, **Klaus S. 2010.** Identification of a cryptic lineage within *Potamon fluviatile* (Herbst) (Crustacea: Brachyura: Potamidae). *Invertebrate Systematics* **24:** 348384–348356.

Kapli P, **Lutteropp S**, **Zhang J**, **Kobert K**, **Pavlidis P**, **Stamatikis A**, **Flouri T. 2017.** Multi-rate Poisson tree processes for single-locus species delimitation under maximum likelihood and Markov chain Monte Carlo. *Bioinformatics* **33:** 1630–1638.

Krauss F. 1843. *Südafrikanischen Crustaceen. Eine Zusammenstellung aller bekannten Malacostraca, Bemerkungen über deren Liebenswiese und geographische Verbreitung, nebst beschreibung und Abbildung mehrer neuen arten*. Stuttgart: E. Schweizerbart'sche Verlagsbuchhandlung.

Linnaeus C. 1758. *Systema naturæ per regna tria naturæ, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis*. Vol. 1. (10th ed.). Stockholm: Laurentius Salvius. pp. [1–4], 1–824.

MacLeay WS. 1838. Brachyurous decapod Crustacea. In: Smith A, ed. *Illustrations of the zoology of South Africa; consisting chiefly of figures and descriptions of the objects of natural history collected during an expedition into the interior of South Africa, in the years 1834, 1835, and 1836; fitted out by "The Cape of Good Hope Association for Exploring Central Africa", (Invertebrates), (1849)*. London: Smith, Elder and Co., 1–75.

Mantelatto FL, **Souza-Carvalho EA**, **Araujo SR**, **Magalhães C. 2022.** Combined multigene and morphological analysis reveal lineage-specific diversification of the Neotropical freshwater crabs of the genus *Fredius* Pretzmann, 1967 (Brachyura, Pseudothelphusidae). *Systematics and Biodiversity* **20:** 1–15.

McDonald DE, **Daniels SR. 2012.** Phylogeography of the Cape velvet worm (Onychophora: *Peripatopsis capensis*) reveals the impact of Pliocene/Pleistocene climatic oscillations on Afromontane forest in the Western Cape, South Africa. *Journal of Evolutionary Biology* **25:** 824–835.

Miller MA, **Pfeiffer W**, **Schwartz T. 2010.** Creating the CIPRES Science Gateway for Inference of large phylogenetic trees. *Proceedings of the Gateway Computing Environments Workshop (GCE)*, 14 November 2010, New Orleans, LA, 1–8.

Milne Edwards H. 1837. Histoires naturelles des Crustaces, comprenant l'anatomie, la physiologie et la classification de ces animaux. **2:** 1–532.

Milne-Edwards A. 1868. Description de quelques crustaces nouveaux. *Nouvelles Annales du Muséum d'Histoire Naturelle* **4:** 69–92.

Ng PKL, **Števcic Z**, **Pretzmann G. 1995.** A revision of the family Deckeniidae Ortmann, 1897 (Crustacea, Decapoda, Brachyura, Potamoidea) from the Seychelles, Indian Ocean. *Journal of Natural History* **29:** 581–600.

Nieto-Montes de Oca A, **Barley AJ**, **Meza-Lazaro RN**, **Garcia-Vazquez UO**, **Zamora-Abrego JG**, **Thomson RC**, **Leache AD. 2017.** Phylogenomics and species delimitation in the knob-scaled lizards of the genus *Xenosaurus* (Squamata: Xenosauridae) using ddRADseq data reveal a substantial underestimation of diversity. *Molecular Phylogenetics and Evolution* **106:** 241–253.

Nixon K, **Wheeler W. 1990.** An amplification of the phylogenetics species concept. *Cladistics* **6:** 221–223.

Ortmann AE. 1896. Das System der Decapoden-Krebse. *Zoologische Jahrbücher, Abteilung für Systematik, Geographie und Biologie de Thiere* **9:** 409–453.

Pante E, **Puillandre N**, **Viricel A**, **Arnaud-Haond S**, **Aurelle D**, **Castelin M**, **Chenuil A**, **Destombe C**, **Forcioli D**, **Valero M**, **Viard F**, **Samadi S. 2015.** Species are hypothesis: avoiding connectivity assessments based on pillars of sand. *Molecular Ecology* **24:** 525–544.

Parmakelis A, **Kotsakiozi P**, **Stathi I**, **Poulikarakou S**, **Fet V. 2013.** Hidden diversity of *Euscorpius* (Scorpiones: Euscorpiidae) in Greece revealed by multilocus species-delimitation approaches. *Biological Journal of the Linnean Society* **110:** 728–748.

Peer N, **Perissinotto R**, **Gouws G**, **Miranda NAF. 2015.** Description of a new species of *Potamonautes* MacLeay 1938, from iSimangaliso Wetland Park, South Africa. *ZooKeys* **503:** 23–43.

Peer N, **Gouws G**, **Lazo-Wasem E**, **Perissinotto R**, **Miranda NAF. 2017.** Redescription of *Potamonautes sidneyi* (Rathbun, 1904) (Decapoda, Potamonautidae) and description of a new species from KwaZulu-Natal, South Africa. *ZooKeys* **657:** 1–28.

Pentinsaari M, **Vos R**, **Mutanen M. 2017.** Algorithmic single-locus species delimitation: effects of sampling effort, variation and nonmonophyly in four methods and 1870 species of beetles. *Molecular Ecology Resources* **17:** 393–404.

Phiri EE, **Daniels SR. 2013.** Hidden in the highlands: the description and phylogenetic positioning of a novel endemic freshwater crab species (Potamonautidae: *Potamonautes*) from Zimbabwe. *Invertebrate Systematics* **27:** 530–539.

Phiri EE, **Daniels SR. 2014.** Disentangling the divergence and cladogenesis in the freshwater crab species (Potamonautidae: *Potamonautes perlatus*) in the Cape Fold Mountains, South Africa, with the description of two novel cryptic lineages. *Zoological Journal of the Linnaean Society* **170:** 310–332.

Phiri EE, **Daniels SR. 2016.** Multilocus coalescent species delimitation reveals widespread cryptic differentiation

among Drakensberg mountain-living freshwater crabs (Decapoda: *Potamonautes*). *Invertebrate Systematics* **30:** 60–74.

Posada D, **Crandall, KA. 1998.** MODELTEST: testing the DNA substitution. *Bioinformatics* **14:** 817–818.

Pretzmann G. 1977. Über einige ostafrikanische Süßwasserkrabben. *Monitore Zoologico Italiano, N.S.* (Suppl. 9) **10:** 233–248.

Puillandre N, **Brouillet S**, **Achaz G. 2021.** ASAP: assemble species by automatic partitioning. *Molecular Ecology Resources* **21:** 609–620.

Rambaut A. 2009. *FigTree*. Available at: http://tree.bio.ed.ac.uk/software/figtree/

Rambaut A, **Suchard MA**, **Xie D**, **Drummond AJ. 2014.** *Tracer v.1.6.* Available at: http://beast.bio.ed.ac.uk/Tracer

Ramírez-Portilla C, **Baird AM**, **Cowman PF**, **Quattrini AM**, **Harii S**, **Sinniger F**, **Flot J-F. 2022.** Solving the coral species delimitation conundrum. *Systematic Biology* **71:** 461–475.

Raphalo EM, **Cole M**, **Daniels SR. 2021a.** Barcoding of South African forest-dwelling snails (Mollusca: Gastropoda) reveals widespread cryptic diversity. *Invertebrate Biology* **140:** e12348.

Raphalo EM, **Cole ML**, **Daniels SR. 2021b.** Climatic oscillations during the Mio/Pliocene epochs induced cladogenesis in the terrestrial snail genus *Gittenedouardia* (Mollusca: Gastropoda: Cerastidae) from South Africa. *Molecular Phylogenetics and Evolution* **155:** 107000.

Rathbun MJ. 1904. Les crabes d'eau douce (Potamonidae). *Nouvelles Archives du Muséum d'Histoire Naturelle de Paris* **6:** 255–312.

Reed SK, **Cumberlidge N. 2004.** Notes on the taxonomy of *Potamonautes obesus* (A. Milne-Edwards, 1868) and *P. calcaratus* (Gordon, 1929) (Brachyura: Potamoidea: Potamonautidae). *Zootaxa* **418:** 1–20.

Reid NM, **Carstens BC. 2012.** Phylogenetic estimation error can decrease the accuracy of species delimitation: a Bayesian implementation of the general mixed Yule-coalescent model. *BMC Evolutionary Biology* **12:** 196.

Ritchie AM, **Lo N**, **Ho SY. 2018.** Examining the sensitivity of molecular species delimitations to the choice of mitochondrial marker. *Organism Diversity &. Evolution* **16:** 467–480.

Ronquist F, **Teslenko M**, **Van Der Mark P**, **Ayres DL**, **Darling A**, **Höhna S**, **Larget B**, **Liu L**, **Suchard MA**, **Huelsenbeck JP. 2012.** MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61:** 539–542.

Shi B, **Pan D**, **Sun Y**, **Liu X**, **Lv X**, **Cumberlidge N**, **Sun H. 2021.** Regional climates shape the biogeographic history of a broadly distributed freshwater crab species complex. *Journal of Biogeography* **48:** 1432–1447.

Stewart BA, **Coke M**, **Cook PA. 1995.** *Potamonautes dentatus*, new species, a fresh-water crab (Brachyura: Potamoidea: Potamonautidae) from KwaZulu-Natal, South Africa. *Journal of Crustacean Biology* **15:** 558–568.

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

Stewart BA, **Cook PA. 1998.** Identification of a new species of river crab (Decapoda: Brachyura: Potamonautidae) from South Africa using morphological and genetic data. *Journal of Crustacean Biology* **18:** 48–61.

Swofford DL. 2002. *PAUP* phylogenetic analysis using parsimony (and other methods), v.4.10.* Champaign: Illinois Natural History Survey.

Taylor PJ, **Kearney T**, **Dalton DL**, **Chakona G**, **Kelly CMR**, **Barker NP. 2020.** Biomes, geology and past climate drive speciation on laminate-toothed rats on South African mountains (Murinae: *Otomys*). *Zoological Journal of the Linnean Society* **189:** 1046–1066.

Thompson JD, **Gibson TJ**, **Plewniak F**, **Jeanmougin F**, **Hiddins DG. 1997.** The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25:** 4876–4882.

Trifinopoulos J, **Nguyen LT**, **von Haeseler A**, **Minh BQ. 2016.** W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Research* **44:** 232–235.

Vitecek S, **Kucinic M**, **Previšic A**, **Živic I**, **Stojanovic K**, **Keresztes L**, **Bálint M**, **Hoppeler F**, **Waringer J**, **Graf W**, **Pauls SU. 2017.** Integrative taxonomy by molecular species delimitation: multi-locus data corroborate a new species of Balkan Drusinae micro-endemics. *BMC Evolutionary Biology* **17:** 129.

Wood LE, **Daniels SR. 2016.** Genetic and morphological evidence for a new mountain-living freshwater crab species (Decapoda: Potamonautidae: *Potamonautes*) from the Western Cape province of South Africa. *Invertebrate Systematics* **30:** 219–230.

Zhang J, **Kapli P**, **Pavlidis P**, **Stamatakis A. 2013.** A general species delimitation method with applications to phylogenetic placements. *Bioinformatics* **29:** 2869–2876.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Maximum likelihood phylogenetic tree topology derived from the 16S rRNA sequence data only. Statistical support for nodes is provided as posterior probability values above nodes (> 0.95 PP) and bootstrap

values below the node (> 75%). An * or # denotes nodal relationships that were not supported (< 0.95 PP/ < 75%). Clade labels correspond to those in Figure 2.

Figure S2. Haplotype network derived from the 16S rRNA sequence data for *P. sidneyi s.l.* representing connectivity amongst all sample localities. Haploclusters dominated by haplotypes from each focal species are defined by grey boxes, despite haplotypes being shared throughout the network.

Figure S3. A, stream habitat of *Potamonautes karooensis* sp. nov., at Asante Sana, Eastern Cape Province, South Africa. B, live image of *P. karooensis* sp. nov., from Asante Sana, Eastern Cape Province, South Africa.

Figure S4. A, stream habitat of *Potamonautes valles* sp. nov., at Muilhuis section (BRCNR), Mpumalanga Province, South Africa. B, habitat of *P. valles* sp. nov., at an unknown locality 30 km north-east of Mbabane, Hhohho Province, Eswatini. C, live image of *P. valles* sp. nov., from Muilhuis section of BRCNR, Mpumalanga Province, South Africa.