DNA barcoding of southern African crustaceans reveals a mix of invasive species and potential cryptic diversity

Bezeng S. Bezeng [1,2,3] *, **Herman F. van der Bank** [1]

1 African Centre for DNA Barcoding, University of Johannesburg, Auckland Park, Johannesburg, South Africa, **2** School of Mathematics & Natural Sciences, University of Venda, P. Thohoyandou, Venda, South Africa, **3** Department of Geography, Environmental Management and Energy Studies, University of Johannesburg, Auckland Park, Johannesburg, South Africa

* bezengsimmy@gmail.com

Abstract

Globally, crustaceans represent one of the most taxonomically diverse and economically important invertebrate group. Notwithstanding, the diversity within this group is poorly known because most crustaceans are often associated with varied habits, forms, sizes and habitats; making species identification by conventional methods extremely challenging. In addition, progress towards understanding the diversity within this group especially in southern Africa has been severely hampered by the declining number of trained taxonomists, the presence of invasive alien species, over exploitation, etc. However, the advent of molecular techniques such as "*DNA barcoding and Metabarcoding*" can accelerate species identification and the discovery of new species. To contribute to the growing body of knowledge on crustacean diversity, we collected data from five southern African countries and used a DNA barcoding approach to build the first DNA barcode reference library for southern African crustaceans. We tested the reliability of this DNA barcode reference library to facilitate species identification using two approaches. We recovered high efficacy of specimen identification/discrimination; supported by both barcode gap and tree-base species identification methods. In addition, we identified alien invasive species and specimens with *'no ID'* in our DNA barcode reference library. The later; highlighting specimens requiring (i) further investigation and/or (ii) the potential presence of cryptic diversity or (iii) misidentifications. This unique data set although with some sampling gaps presents many opportunities for exploring the effect and extent of invasive alien species, the role of the pet trade as a pathway for crustacean species introduction into novel environments, sea food authentication, phylogenetic relationships within the larger crustacean groupings and the discovery of new species.

Introduction

Biodiversity loss has emerged as a global concern over the last decades, partly due to human activities [1]. As a result, many nations are taking stringent measures to mitigate this loss,

especially in the face of global change. However, current measures have been limited due in part to the unknown extent of global biodiversity, with earlier studies showing that only an approximately 15–18% of all living organisms have been formally described to species level [2–3]. On the other hand, a more recent study using new ecological rules for how biodiversity relates to abundance suggests that there are likely to be at least 1 to 6 billion species on earth see [4] and references therein. Therefore, much progress has been made over the years by taxonomists to discover and describe new species globally, although progress towards any meaningful outcome has been hampered by the declining number of trained taxonomists, and the availability of cryptic species amongst others [5–6]. In the face of declining numbers of trained taxonomists, alternative methods that provide rapid and accurate identification of living organisms have been proposed. Specifically, [7] proposed the use of a short and standardize portion of the genome otherwise known as "DNA Barcodes" for the identification of living organisms. Over a decade and a half since DNA barcoding techniques for specimen identification was established, this method and its associated applications have become increasingly popular in biodiversity science see review by [8] and references therein. For example, DNA barcoding techniques have been widely used to (i) evaluate commercial food product labelling accuracy [9], (ii) identify threatened animal product sold for traditional medicine [10] and particularly wildlife crime [11] etc. Of all these applications, the most prominent has been to document and discover biodiversity within various animal groups including insects, molluscs, fish, mammals etc. [12–14]. However, studies employing the utility of DNA barcodes for species discrimination within crustaceans are still limited but see [15–20]. More so, even with the advent of DNA metabarcoding; a method for assessing biodiversity from environmental samples through bulk DNA extraction, amplification and high throughput sequencing of multiple taxa [21], Carcinologists have not fully taken advantage of this new technique to document and discover new species but see [22]. Yet, crustaceans represent one of the most taxonomically diverse, ecological and economical important invertebrate group [9, 16–17, 20]. For example, crabs, crayfish, lobsters, prawns and shrimps are some of the most expensive and sought-after foods globally. Given their importance, many crustaceans remain under increasing threat from human activities; with the introduction of new species into novel environments increasingly being recognised as a major threat. Such introductions have caused some crustacean species to become established and hence invasive, causing significant negative ecological and economic impacts [23–28]. For example, applying a Generic Impact Scoring System (GISS), a recent study identified the Chinese mitten crab, *Eriocheir sinensis* (Milne-Edwards, 1853) as the most impacting invasive species in Europe based on six environmental and socio-economic categories [29] but see also [30–31] for specific impact of *Balanus glandula* (Darwin, 1854) in South Africa]. With the increasing threats and their global importance, our knowledge within this group globally is still very limited due in part to the fact that most crustaceans are associated with varied habits, forms, sizes and habitats ranging from marine, brackish, to freshwater environments. As a result, crustaceans exhibit a remarkable morphological diversity, involving many potentially cryptic species thus making species identification a challenge. Although previous studies from elsewhere in the world on crustaceans have shown the utility of DNA barcoding techniques for species discrimination in both marine and freshwater systems [15–20, 32], in southern Africa, our knowledge of the crustacean fauna remains limited but see [9]. Therefore, in this study, we aim to build the first DNA barcode reference library of crustacean taxa found in southern Africa (i.e. Angola, Mozambique, Namibia, South Africa, and Swaziland) to facilitate species identification. This reference DNA barcoding library in addition to facilitating species identification will hold enormous potential for the seafood industry, invasive species management, fishing industry etc. in southern Africa and beyond.

Materials and methods

Field collections

Samples of crustaceans were collected between April 2010 –February 2012 along the southern African coastline and in deep water using trawler boats. Additional samples were collected from freshwater systems (e.g. the Cunenne River mouth). Individual specimens were collected under permit number MAM 18 066202 from the Department of Agriculture, Forestry and Fisheries, South Africa and our studies did not involve endangered or protected species. Specimens were collected from five southern African countries including; Angola, Mozambique, Namibia, Robben Island (South Africa) and Swaziland. Additional samples were collected on a humpback whale; washed off the western coast near Britannia Bay (South Africa; **Fig 1**). Individual specimens were collected under permit number MAM 18 066202 from the Department of Agriculture, Forestry and Fisheries, South Africa.

Morphological species identification was facilitated following expert knowledge, published field guides [25, 33]. All collection details have been logged in public repositories. Specifically,
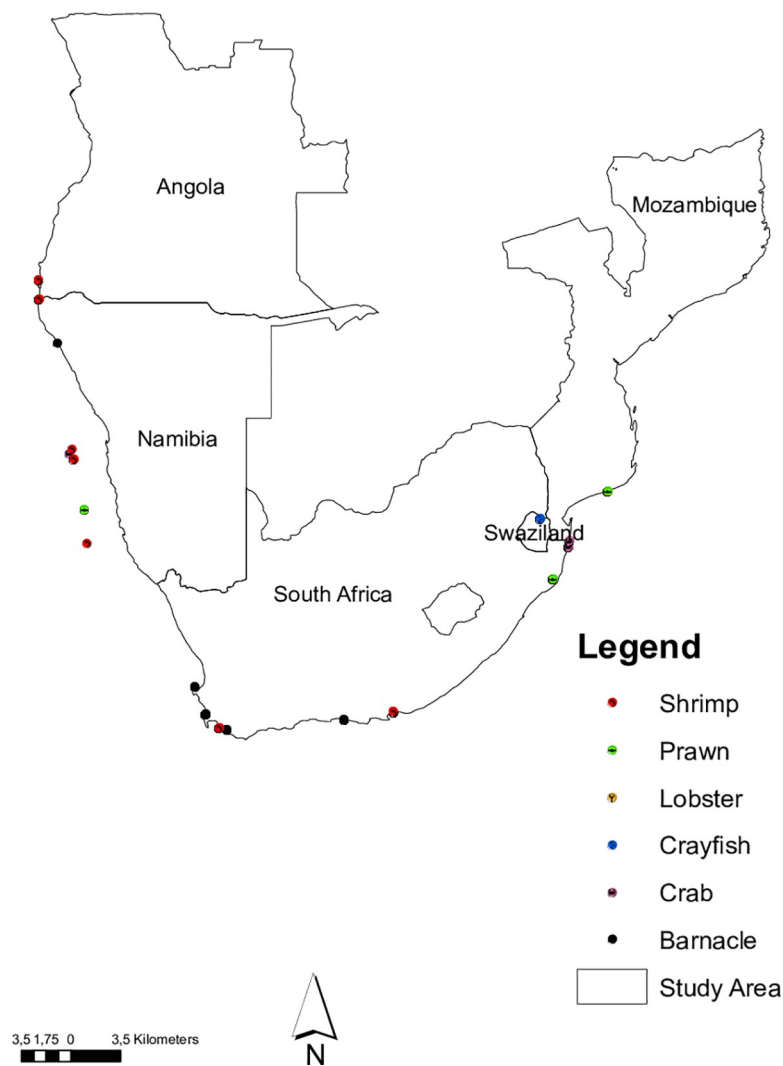
Fig 1. Southern African countries and collection efforts of crustaceans included in our study.

https://doi.org/10.1371/journal.pone.0222047.g001

collection details including GPS coordinates, altitude and photographs were deposited in Barcode of Life Data Systems (BOLD; www.boldsystems.org), under the project code HVDBC-Southern African Crustacea.

DNA extraction, amplification and sequencing

DNA extraction, polymerase chain reactions (PCR) and sequencing of the *COI* gene region (i.e. animal DNA barcode) were done at the Canadian Centre for DNA Barcoding (CCDB) and the African Centre for DNA Barcoding (ACDB) using the primer combination; C_Lep-FolF and C_LepFolR [34] and following standard protocol as outlined in [35]. For all samples that were successfully amplified, GenBank accession numbers, BOLD process identification numbers, authorities and voucher information are available online in BOLD under the project code HVDBC- Southern African Crustacea.

Data analyses

Sequence alignment was done using Multiple Sequence Comparison by Log-Expectation MUSCLE version. 3.8.31; [36] and subsequently manually adjusted where necessary. We included in the DNA matrix additional *COI* sequences; including an outgroup retrieved from GenBank database (S1 Table) to test the reliability of our reference library to discriminate amongst species.

Phylogeny reconstruction

Using the aligned sequence data, we reconstructed the first southern African regional phylogeny for crustaceans. The tree was reconstructed using maximum parsimony (MP) as implemented in PAUP* version. 4.0 [37]. Tree analysis was done by running heuristic searches with 1 000 random sequence additions but keeping only 10 trees per replicate to reduce time spent on branch swapping in each replicate. Tree bisection-reconnection was done with all character transformations treated as equally likely i.e. Fitch parsimony; [38]. MP searches and bootstrap resampling [39] were done to assess node support. Bootstrap support values ranged from: >95% = high support, 70–95% = moderate support and <70% = poor support [39–40]. We used a sea spider, *Nymphon charcoti* (Bouvier 1911) (GenBank ID number: FJ969364), as outgroup to establish monophyly of the species.

Species identification

We used a combination of approaches to ensure accurate identification of all crustacean species collected. First, we confirmed the identity of the species using morphological characters as per expert opinion and illustrated field guides e.g. [25, 33]. Second, specimen authenticity were verified by BLAST algorithm on GenBank. Last, we used a tree-base identification method to determine the grouping of each species with its close relative(s) on the phylogenetic tree reconstructed [41]. Following this approach, DNA sequences are simulated on the phylogenetic trees and when the query's conspecific sequences were included in the reference alignment, the rate of positive identification is related to the degree to which different species were genetically differentiated.

Barcode gap analysis

Using the southern African crustacean phylogeny reconstructed, we evaluated if there exist any significant difference between the mean intra- specific and the mean inter-specific genetic distance based on the Kimura 2-parameter model (K2P) [42–43] for more details on

Table 1. Number of specimens representing families, genera, and species included in our reference library.

Native Crustaceans	Families	Genera	Specimens (Unique species)
	16	**24**	**149 (28)**
Alien invasive Crustaceans	5	5	25 (5)

techniques. Lastly, we ran a sequence based simulation analysis as implemented in the R Library SPIDER [44] to test the reliability of the southern African crustacean sequence library to correctly identify species using two criteria; Best Close Match (BCM) and All Species Barcode (ASB). The BCM criterion assigns correct identifications to the closest match regardless of the distance, whereas the ASB criterion simulates the BOLD ID engine by applying a threshold and querying all the sequences within BOLD. First, a species is correctly identified when all the matching sequences below the threshold are conspecific and results are reported as correct when consistent with prior morphological identifications, otherwise the result is incorrect. Second, using the ASB criterion, a query may be reported as ambiguous if sequences divergences of different species are below the threshold (ASB) or sequences from different species are the closest match below threshold (BCM). Last, a query may result in no ID if no match is found below the defined threshold see also [17]. For both BCM and ASB, we used the standard thresholds for BOLD ID engine i.e. 1% K2P; [45]. Additionally, a second threshold was used that minimizes the cumulative identification errors; as implemented in the function 'threshVal' in SPIDER. In this approach we identified the sum of false positive (no conspecific matches within threshold of query) and false negative (sequences from multiple species within threshold).

Results

The DNA barcoding library for southern African crustaceans consist of 174 specimens including seven specimens downloaded from GenBank (S1 Table). We also included a sea spider (*Nymphon charcoti* (Bouvier 1911)) as outgroup. The specimens were distributed as follows: Angola (n = 5) Mozambique (n = 49), Namibia (n = 24), South Africa (n = 84), Swaziland (n = 5) and GenBank (n = 8). Additionally, we identified five invasive species; the whale and rabbit-ear barnacles; *Coronula diadema* (Linnaeus 1767) and *Conchoderma auritum* (Linnaeus 1767), Pacific barnacle; *Balanus glandula* (Darwin 1854), African river prawn; *Macrobrachium vollenhovenii* (Herklots 1857), and redclaw crayfish; *Cherax quadricarinatus* (von Martens 1868). These specimens were spread across 21 families, 29 genera and 33 unique species (Table 1).

The southern Africa regional phylogeny for crustaceans representing 174 species was well supported with moderate to high bootstrap values (see Fig 2A–2E). Additionally, we recovered high percentage identification success for southern African crustaceans using both field guides and BLAST algorithm (see S1 Table).

Barcoding gap analysis

Our barcode gap analyses of all individuals belonging to 33 unique species and 21 crustacean families (see Table 1) reveals statistically significant differences in K2P values between the mean intraspecific and interspecific comparisons (Mann–Whitney U-test: U = 1106, P < 0.001; Fig 3). From the SPIDER analysis and using the BCM criterion, we obtained 143 correct and 5 incorrect identifications (i.e. correct and incorrect identifications indicate positive and negative outcome for this test), 6 ambiguous identifications (i.e. an ambiguous identification refers to the presence of both correct and incorrect identifications or more than one
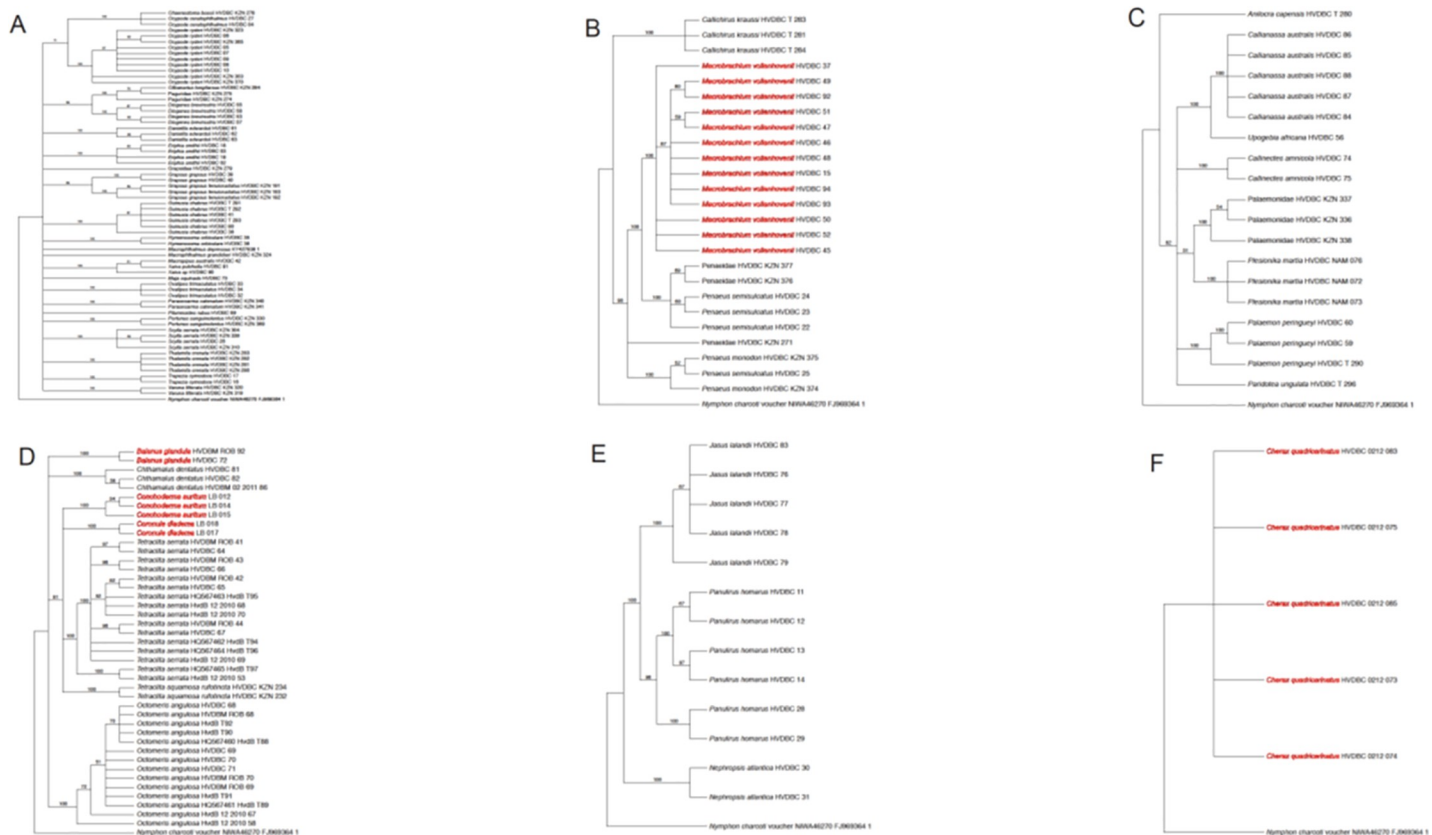
Fig 2. Regional phylogeny of southern African crustaceans (A) Regional phylogeny of crabs (B) Regional phylogeny of prawns (C) Regional phylogeny of shrimps (D) Regional phylogeny of barnacles (E) Regional phylogeny of lobsters (F) Regional phylogeny of crayfish. Species in red indicates the distribution of invasive species on the phylogenetic tree.

equally close matched species with different identification including the correct one) and 20 no ID (i.e. if query sequence matches were found below the proposed threshold). Using the ASB criterion, we obtained 139 correct and 4 incorrect identifications, 11 ambiguous identifications and 20 no ID. In all analyses, 27 identifications were associated to singletons (i.e. without any conspecific sequences to match). After removing these singletons, incorrect identifications were reduced to two using both criteria.

Discussion

Crustaceans form one of the most biologically diverse, species rich, nutrient recyclers and economically important arthropod species on Earth. [46] estimated >17,600 living and extinct species of crustaceans, comprising of barnacles, crabs, lobsters, prawns, shrimps etc. but some studies indicate that the actual species diversity within this group of organisms is still several times higher than is presently accepted [47–49]. For example, a more recent article estimates that there exist between 50,000–67,000 known crustaceans globally but scientists estimate the total number of crustaceans to be ten-fold greater than this [50]. This high diversity within crustaceans makes species identification extremely challenging. Although a few morphological characters (i.e. tail length, gill type and number of chelae) have been used in previous studies as key diagnostic features to identify and classify crustaceans [51–53], progress has been slow due to the declining number of trained taxonomists [6]. Nonetheless, the use of modern
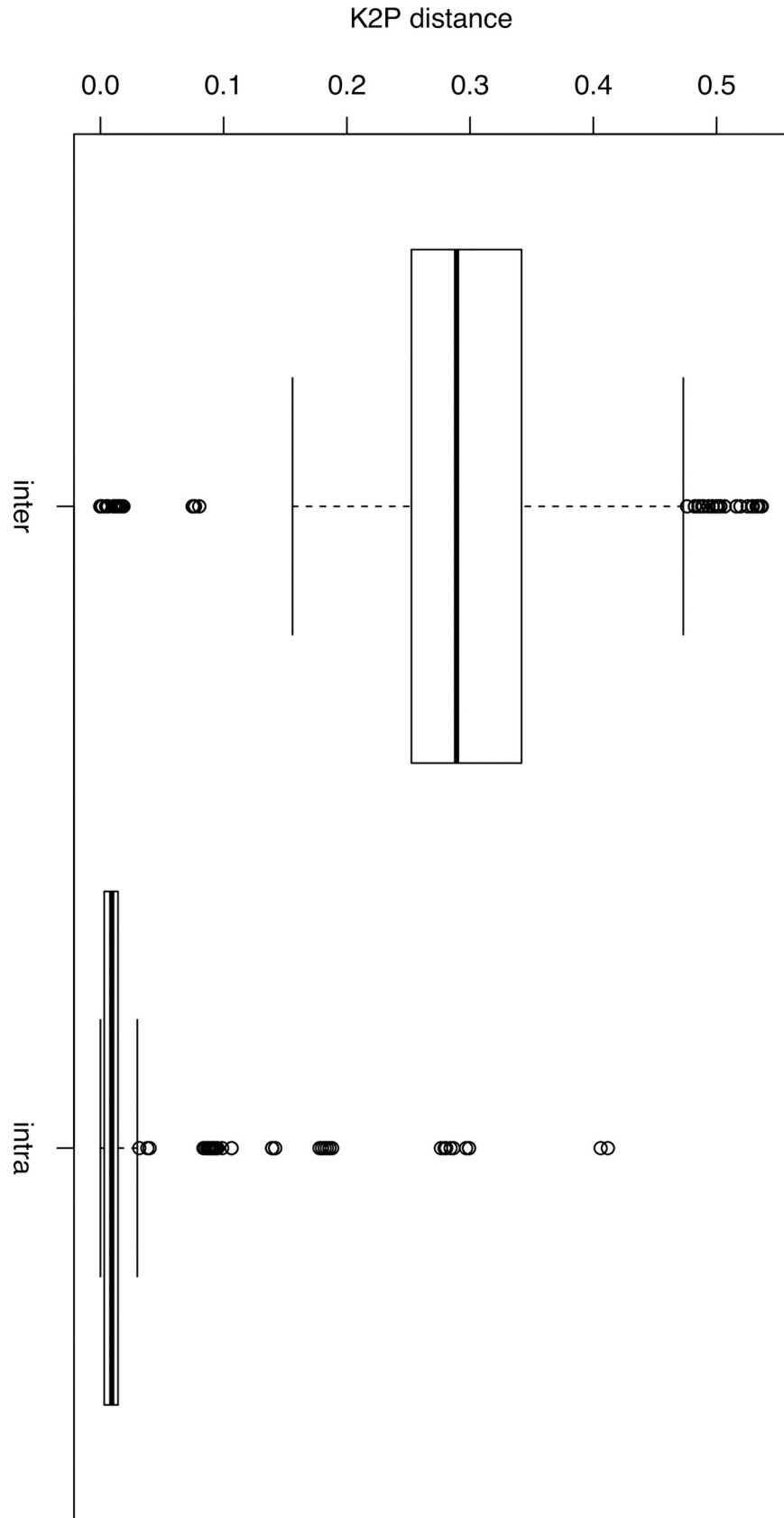
Fig 3. Significantly different genetic distances between and within crustacean species. Box plots represent intraspecific and interspecific genetic distances calculated using Kimura 2-parameter (K2P) model for all species and error bars are interquartile ranges.

techniques such as DNA barcoding and metabarcoding can accelerate species identification and discovery [20–21, 35]. In this study, we present the first large scale DNA barcoding library published for southern African crustacean species. From this DNA barcode library, many potential cryptic and alien invasive species were identified, including the whale and rabbit-ear barnacles; *Coronula diadema* (Linnaeus 1767) and *Conchoderma auritum* (Linnaeus 1767) respectively collected from a dead worldwide occurring humpback whale; *Megaptera novaeangliae* (Borowski 1781), washed off the western coast near Britannia Bay (South Africa), Pacific barnacle; *Balanus glandula* (Darwin 1854), African river prawn; *Macrobrachium vollenhovenii* (Herklots 1857), and the Australian redclaw crayfish; *Cherax quadricarinatus* (von Martens 1868) see also [26, 28, 30]. The proliferation of alien invasive crustaceans across water bodies in Africa in general and southern Africa has also been confirmed by some earlier exploratory studies using both taxonomic and DNA barcoding techniques [25–28, 30]. The mere presence of these species and many more undetected species calls for renewed efforts to better understand their biology in these non-native ranges through research; prioritising alien invasive distribution extents, habitat preferences, impacts on native species and local aquatic ecosystems, and management and control. By so doing, rapid response alien mitigation strategies will be developed, which will go long way to minimise the negative impacts of these species on the rich native species diversity. Such strategies will help alien species managers to better prioritise their control and where possible their eradication. Although sampling efforts were concentrated in five southern African countries (see **Fig 1**), we believe the diversity of alien and native species is much higher than current knowledge from the region allows. Exploring the regional phylogeny of southern African crustaceans revealed patterns similar to other studies [54] and references therein. Many previous studies have attempted to resolve the phylogeny of crustaceans, but several challenges exist with many controversies surrounding the internal relationships amongst major taxonomic groups [53–62]. Results from the studies above, including ours (**Fig 2**), indicates that the controversies in resolving the phylogeny of crustaceans remains and the hope to use a single gene to address this challenge is not yet satisfied. This southern African crustacean reference library represents a first step towards using DNA barcoding techniques for the identification of crustacean species in this region. Using the different approaches to identify species (field guide, expert opinion and BLAST), we recovered high efficacy (mean bootstrap support value = 97%) of specimen identification, which was supported by simulations using SPIDER analysis; based on BM and ASB criteria. Also, our reference library reveals the presence of barcoding gap, with the mean interspecific genetic distance being significantly higher than the mean intraspecific genetic distances ($P < 0.001$; **Fig 3**). Furthermore, the mere presence of specimen with *no ID* in our sequence reference library for southern African crustaceans using both criteria mentioned above, highlights specimens requiring (i) further investigation and/or (ii) the potential presence of cryptic diversity or (iii) misidentifications. However, this result should be interpreted with care and not to be confused with "species delimitation" [63]. Notwithstanding, some concepts applied here overlap with those used for species delimitation, but results from our simulation analysis exclusively tested the performance of a molecular dataset for identifying represented species.

Implication for conservation and way forward

Crustaceans consist of one of the most diverse groups of invertebrates with species of high economic values (e.g. crabs, lobsters, and shrimps) and forms the basis of widespread harvesting

around the world. With their broad distribution, crustaceans are found in almost all aquatic environments and many species like some crabs have successfully inhabited terrestrial environments. Due to this diversity, species identification has proven challenging for many individuals as very few morphological characters can clearly distinguish between them [52]. This is further compounded by the increasing decline in trained taxonomists around the world and especially in southern Africa [6]. As a result, alternative identification methods have been proposed using molecular sequencing techniques. Specifically, DNA barcoding has been used for over a decade and a half [7–8]. In this study, we use DNA barcoding to generate the first large scale reference library for southern African crustaceans. This unique data set presents many opportunities for exploring the effect and extent of invasive alien species, the role of the pet trade as a pathway for crustacean species introduction into new environment, sea food authentication, phylogenetic relationships within the larger crustacean groupings and the discovery of new species [9, 23–24, 26, 53, 64]. More exploratory studies are encouraged especially from the less known African waters; taking advantage of new developments in the field of biodiversity science (artificial intelligence, remote sensing, DNA metabarcoding etc.) to fill the data and knowledge gaps identified in this study.

Conclusions

The data set presented here, although with some sampling gaps, represents an important first step towards establishing a complete DNA reference library for southern African crustaceans. Even though there exist some challenges in utilising DNA barcoding techniques, for example its high sensitivity to environmental contaminants, our reference library holds great potential for species identification, invasive species biomonitoring, sea food authentication and taxonomic revision of major groups amongst others. Notwithstanding, with the advancements in DNA barcoding and metabarcoding technologies [21], crustacean surveys in particular and the biomonitoring of alien and native species in general will proceed faster since metabarcoding techniques have been successful at detecting taxa even at low abundance from environmental samples, whereas there were unnoticed by conventional taxonomic and DNA barcoding methods. This is reflected in our study area, where we found some alien invasive species including *Balanus glandula* (an invasive pacific barnacle arguably introduced through ballast water from ships coming from the Pacific North America).

Supporting information

S1 Table. Supplementary table highlighting all sequence IDs generated by this study and downloaded from GenBank and percentage specimen identification.
(XLSX)

Acknowledgments

Author Contributions

Conceptualization: Herman F. van der Bank.

Formal analysis: Bezeng S. Bezeng.

Funding acquisition: Herman F. van der Bank.

Investigation: Bezeng S. Bezeng, Herman F. van der Bank.

Methodology: Bezeng S. Bezeng, Herman F. van der Bank.

Supervision: Herman F. van der Bank.

Writing – original draft: Bezeng S. Bezeng, Herman F. van der Bank.

Writing – review & editing: Bezeng S. Bezeng, Herman F. van der Bank.

References

1. Pimm SL, Russell GJ, Gittleman JL, Brooks TM. The future of biodiversity. Science. 1995; 269, 347–350. https://doi.org/10.1126/science.269.5222.347 PMID: 17841251

2. Wilson EO. The encyclopedia of life. Trends Ecol Evol. 2003; 18, 77–80.

3. Mora C, Tittensor DP, Adl S, Simpson AGB, Worm B. How Many Species Are There on Earth and in the Ocean? PLoS Biol. 2011; 9, e1001127. https://doi.org/10.1371/journal.pbio.1001127 PMID: 21886479

4. Larsen BB, Miller EC, Rhodes MK, Wiens JJ. Inordinate Fondness Multiplied and Redistributed: the Number of Species on Earth and the New Pie of Life. Q Rev Biol. 2017; 92, 229–265.

5. Gaston KJ, May RM. Taxonomy of taxonomists. Nature. 1992; 356, 281–282.

6. Hopkins GW, Freckleton RP. Declines in the numbers of amateur and professional taxonomists: implications for conservation. Anim. Conserv. 2002; 5, 245–249. https://doi.org/10.1017/S1367943002002299

7. Hebert PDN, Ratnasingham S, deWaard JR. Barcoding animal life: cytochrome c oxidase subunit 1divergences among closely related species. Proc Biol Sci. 2003; 270, S96–S99. https://doi.org/10.1098/rsbl.2003.0025 PMID: 12952648

8. Bezeng BS, Davies TJ, Daru BH, Kabongo RM, Maurin O, Yessoufou K, et al. Ten years of plant DNA barcoding at the African Centre for DNA Barcoding. Genome. 2017; https://doi.org/10.1139/gen-2016-0198 PMID: 28340301

9. Cawthorn DM, Hoffman LC. Deceit with decapods? Evaluating labelling accuracy of crustacean products in South Africa. Food Control. 2017; 73, 741–753.

10. Yan D, Luo JY, Han YM, Peng C, Dong XP, Chen SL, et al. Forensic DNA Barcoding and Bio-Response Studies of Animal Horn Products Used in Traditional Medicine. PLoS ONE. 2013; 8, e55854. https://doi.org/10.1371/journal.pone.0055854 PMID: 23409064

11. Staats M, Arulandhu AJ, Gravendeel B, Holst-Jensen A, Scholtens I, Peelen T, et al. Advances in DNA metabarcoding for food and wildlife forensic species identification. Anal. Bioanal. Chem. 2016; 408, 4615–4630. https://doi.org/10.1007/s00216-016-9595-8 PMID: 27178552

12. Ward RD, Hanner R, Hebert PD. The campaign to DNA barcode all fishes, FISH-BOL. J Fish Biol. 2009; 74, 329–356. https://doi.org/10.1111/j.1095-8649.2008.02080.x PMID: 20735564

13. Schmidt S, Schmid-Egger C, Morinière J, Haszprunar G, Hebert PDN. DNA barcoding largely supports 250 years of classical taxonomy: identifications for Central European bees (Hymenoptera, Apoidea partim). Mol. Ecol. Resour. 2015; 15, 985–1000. https://doi.org/10.1111/1755-0998.12363 PMID: 25588628

14. Van der Bank H, Greenfield R. A pioneer survey and DNA barcoding of some commonly found gastropod molluscs on Robben Island. ZooKeys. 2015; 481, 15–23. https://doi.org/10.3897/zookeys.481.8188 PMID: 25685029

15. Costa FO, deWaard JR, Boutillier J, Ratnasingham S, Dooh RT, Hajibabaei M, et al. Biological identifications through DNA barcodes: the case of the Crustacea. Can J Fish Aquat Sci. 2007; 64, 272–295. https://doi.org/10.1139/f07-008

16. Radulovici AE, Sainte-Marie B, Dufresne F. DNA barcoding of marine crustaceans from the estuary and Gulf of St Lawrence: a regional-scale approach. Mol. Ecol. Resour. 2009; 9, 181–187. https://doi.org/10.1111/j.1755-0998.2009.02643.x PMID: 21564977

17. Raupach MJ, Barco A, Steinke D, Beermann J, Laakmann S, Mohrbeck I, et al. The Application of DNA Barcodes for the Identification of Marine Crustaceans from the North Sea and Adjacent Regions. PLoS ONE. 2015; 10, e0139421. https://doi.org/10.1371/journal.pone.0139421 PMID: 26417993

18. Raupach MJ, Radulovici AE. Looking back on a decade of barcoding crustaceans. ZooKeys. 2015; 539, 53–81. https://doi.org/10.3897/zookeys.539.6530 PMID: 26798245

19. Witt JDS, Threloff DL, Hebert PDN. DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. Mol Ecol. 2006; 172, 17–32.

20. Bekker EI, Karabanov DP, Galimov YR, Kotov AA. DNA Barcoding Reveals High Cryptic Diversity in the North Eurasian Moina Species (Crustacea: Cladocera). PLoS ONE. 2016; 11, e0161737. https://doi.org/10.1371/journal.pone.0161737 PMID: 27556403

21. Hajibabaei M, Shokralla S, Zhou X, Singer GAC, Baird DJ. Environmental Barcoding: A Next-Generation Sequencing Approach for Biomonitoring Applications Using River Benthos. PLoS ONE. 2011; 6, e17497. https://doi.org/10.1371/journal.pone.0017497 PMID: 21533287

22. Hollatz C, Leite BR, Lobo J, Froufe H,Egas C, Costa FO. Priming of a DNA metabarcoding approach for species identification and inventory in marine macrobenthic communities. Genome. 2017; 60, 260–271. https://doi.org/10.1139/gen-2015-0220 PMID: 28145743

23. Molnar JL, Gamboa RL, Revenga C, Spalding MD. Assessing the global threat of invasive species to marine biodiversity. Front. Ecol. Environ. 2008; 6, 485–492, https://doi.org/10.1890/070064

24. Nunes AL, Zengeya TA, Measey GJ and Weyl OLF. Freshwater crayfish invasions in South Africa: past, present and potential future. Afr J Aquat Sci. 2017; 42, 309–323.

25. Picker MD, Griffiths CL. Alien and Invasive Animals—A South African Perspective: Casciola J. (Ed). 2011; Struik-Random House Publishers, Cape Town, 2011. 248pp.

26. Petersen RM, Hoffman AC, Kotze P, Marr SM. First record of the invasive Australian redclaw crayfish *Cherax quadricarinatus* (von Martens, 1868) in the Crocodile River, Kruger National Park, South Africa. Koedoe. 2017; 59, a1435. https://doi.org/10.4102/koedoe. v59i1.1435

27. Kuguru B, Groeneveld J, Singh S, Mchomvu B. First record of giant freshwater prawn *Macrobrachium rosenbergii* (de Man, 1879) from smallscale fisheries in East Africa, confirmed with DNA barcoding. BioInvasions Rec. 2019; 8, 379–391, https://doi.org/10.3391/bir.2019.8.2.19

28. Kerckhof F, De Mesel I, Degraer S. First European record of the invasive barnacle *Balanus glandula* Darwin, 1854. BioInvasions Rec. 2018; 7, 21–31.

29. Laverty C, Nentwig W, Dick JTA, Lucy FE. Alien aquatics in Europe: assessing the relative environmental and socioeconomic impacts of invasive aquatic macroinvertebrates and other taxa. Manag Biol Invasion. 2015; 6, 341–350.

30. Laird MC, Griffiths CL. Present distribution and abundance of the introduced barnacle *Balanus glandula* Darwin, 1854 in South Africa. Afr. J. Mar. Sci. 2008; 30, 93–100, https://doi.org/10.2989/AJMS.2008.30.1.9.459

31. Simon-Blecher N, Granevitze Z, Achituv Y. 2008. *Balanus glandula*: from North-west America to the west coast of South Africa. Afr. J. Mar. Sci. 2008; 30, 85–92. https://doi.org/10.2989/AJMS.2008.30.1.8.458

32. Bucklin A, Wiebe PH, Smolenack SB, Copley NJ, Beaudet JG, Bonner KG, et al. DNA barcodes for species identification of euphausiids (Euphausiacea, Crustacea). J Plankton Res 2007; 29: 483–493.

33. Branch GM, Griffiths CL, Branch ML, Beckley LE. Two Oceans: A guide to the marine life of southern Africa. 2010; Struik Nature, South Africa, 456pp.

34. Hebert PDN, Braukmann TWA, Prosser SWJ, Ratnasingham S, de Waard JR, Ivanova NV, et al. A Sequel to Sanger: Amplicon Sequencing That Scales. bioRxiv. 2017; http://dx.doi.org/10.1101/191619.

35. Hajibabaei M, De Waard JR, Ivanova NV, Ratnasingham S, Dooh RT, Kirk SL, et al. Critical factors for assembling a high volume of DNA barcodes. Philos Trans R Soc London B Biol Sci. 2005; 360, 1959–1967. https://doi.org/10.1098/rstb.2005.1727 PMID: 16214753

36. Edgar RC. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; 32, 1792–1797. https://doi.org/10.1093/nar/gkh340 PMID: 15034147

37. Swofford DL. PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b10. 2003; Sinauer, Sunderland, Massachusetts

38. Fitch WM. Towards defining the course of evolution: minimum change for a specific tree topology. Syst Biol. 1971; 20, 406–416.

39. Phylogenies Felsenstein J. and the comparative method. Am Nat. 1985; 125, 1–15.

40. Soltis PS, Soltis DE. Applying the Bootstrap in Phylogeny Reconstruction. Stat Sci. 2003; 18, 256–267

41. Ross HA, Murugan S, Li WS. Testing the Reliability of Genetic Methods of Species Identification via Simulation. Syst. Biol. 2008; 57, 216–230. https://doi.org/10.1080/10635150802032990 PMID: 18398767

42. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 1980; 16, 111–120. https://doi.org/10.1007/bf01731581 PMID: 7463489

43. Čandek K, Kuntner M. DNA barcoding gap: reliable species identification over morphological and geographical scales. Mol. Ecol. Resour. 2015; 15, 268–277. https://doi.org/10.1111/1755-0998.12304 PMID: 25042335

44. Brown SDJ, Collins RA, Boyer S, Lefort M-C, Malumbres-Olarte J, Vink CJ, et al. SPIDER: An R package for the analysis of species identity and evolution, with particular reference to DNA barcoding. Mol Ecol Resour. 2012; 12, 562–565. https://doi.org/10.1111/j.1755-0998.2011.03108.x PMID: 22243808

45. Ratnasingham S, Hebert PDN. BOLD: The Barcode of Life Data Systems. Mol Ecol Notes. 2007; 7, 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x PMCID: PMC1890991 PMID: 18784790.

46. De Grave SD, Pentcheff ND, Ahyong ST, et al. A classification of living and fossil genera of decapod crustaceans. Raffles Bull. Zool. Suppl. 2009; 21, 1–109.

47. De Grave SD, Cai Y, Anker A. Global diversity of shrimps (Crustacea: Decapoda: Caridea) in freshwater. Hydrobiologia. 2008; 595, 287–293

48. Poore GCB, Bruce NL. Global Diversity of Marine Isopods (Except Asellota and Crustacean Symbionts). PLoS ONE. 2012; 7, e43529. https://doi.org/10.1371/journal.pone.0043529 PMID: 22952700

49. Griiths CL, Landschof J, Atkinson LJ. Phylum Arthropoda In: Atkinson LJand Sink KJ(eds) Field Guide to the Ofshore Marine Invertebrates of South Africa, Malachite Marketing and Media, Pretoria. 2018. pp. 133–226.

50. Bruce N, MacDiarmid A. Crabs, crayfish and other crustaceans', Te Ara—the Encyclopedia of New Zealand, http://www.TeAra.govt.nz/en/crabs-crayfish-and-other-crustaceans/print. 2006. (Last accessed 12 June 2019).

51. Scholtz G, Richter S. Phylogenetic systematics of the reptantian Decapoda (Crustacea, Malacostraca). Zool. J. Lin. Soc. 1995; 113, 289–328.

52. Schram FR. Phylogeny of decapods: moving towards a consensus. Hydrobiologia. 2001; 449, 1–20.

53. Dixon CJ, Ahyong ST, Schram FR. A new hypothesis of decapod phylogeny. Crustaceana. 2003; 76, 935–975.

54. Shen B, Scholtz B. Mitogenomic analysis of decapod crustacean phylogeny corroborates traditional views on their relationships. Mol. Phylogenet. Evol. 2013; 66, 776–789 https://doi.org/10.1016/j.ympev.2012.11.002 PMID: 23202543

55. Ahyong ST, O'Meally D. Phylogeny of the Decapoda Reptantia: resolution using three molecular loci and morphology. Raffles Bull. Zool 2004; 52, 673–693.

56. Porter ML, Pérez-Losada M, Crandall KA. Model-based multi-locus estimation of decapod phylogeny and divergence times. Mol. Phylogenet. Evol. 2005; 37, 355–369. https://doi.org/10.1016/j.ympev.2005.06.021 PMID: 16112880

57. Ahyong ST. Phylogeny of the clawed lobsters (Decapoda Homarida). Zootaxa 2006; 1109, 1–14.

58. Ahyong ST, Lai JCY, Sharkey D, Colgan DJ, Ng PKL. Phylogenetics of the brachyuran crabs (Crustacea: Decapoda): the status of Podotremata based on small subunit nuclear ribosomal RNA. Mol. Phylogenet. Evol 2007; 45, 576–586. https://doi.org/10.1016/j.ympev.2007.03.022 PMID: 17548212

59. Tsang LM, Ma KY, Ahyong ST, Chan TY, Chu KH. Phylogeny of Decapoda using two nuclear protein-coding genes: origin and evolution of the Reptantia. Mol. Phylogenet. Evol. 2008; 48, 359–368. https://doi.org/10.1016/j.ympev.2008.04.009 PMID: 18501643

60. Ahyong ST, Schnabel KE, Maas EW. Anomuran phylogeny: new insights from molecular data. In: Martin JW, Felder DL, Crandall KA. (Eds.), Decapod Crustacean Phylogenetics (Crustacean Issues 18). 2009; CRC Press, Boca Raton, pp.399–414.

61. Bracken HD, Toon A, Felder DL, Martin JW, Finley M, Rasmussen J, et al. The decapod tree of life: compiling the data and moving toward a consensus of decapod evolution. Arthr. Syst. Phylog. 2009; 67, 99–116.

62. Toon A, Finley M, Staples J, Crandall KA. Decapod phylogenetics and molecular evolution. In: Martin JW, Felder DL, Crandall KA. (Eds.), Decapod Crustacean Phylogenetics (Crustacean Issues 18). 2009; CRC Press, Boca Raton, pp. 15–30.

63. Collins RA, Cruickshank RH. The seven deadly sins of DNA barcoding. Mol Ecol Resour. 2012; 13, 969–975. https://doi.org/10.1111/1755-0998.12046 PMID: 23280099

64. Uderbayev T, Patoka J, Beisembayev R, Petrtyl M, Bláha M, Kouba A. Risk assessment of pet-traded decapod crustaceans in the Republic of Kazakhstan, the leading country in Central Asia. Knowl. Manag. Aquat. Ecosyst. 2017; 418, 30.