KOEDOE - African Protected Area Conservation and Science
ISSN: (Online) 2071-0771, (Print) 0075-6458

Probing hidden diversity to enhance conservation of the endangered narrow-range endemic Eastern Cape rocky, *Sandelia bainsii* (Castelnau 1861)

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Dates:
Received: 03 Mar. 2020
Accepted: 22 July 2020
Published: 29 Sept. 2020

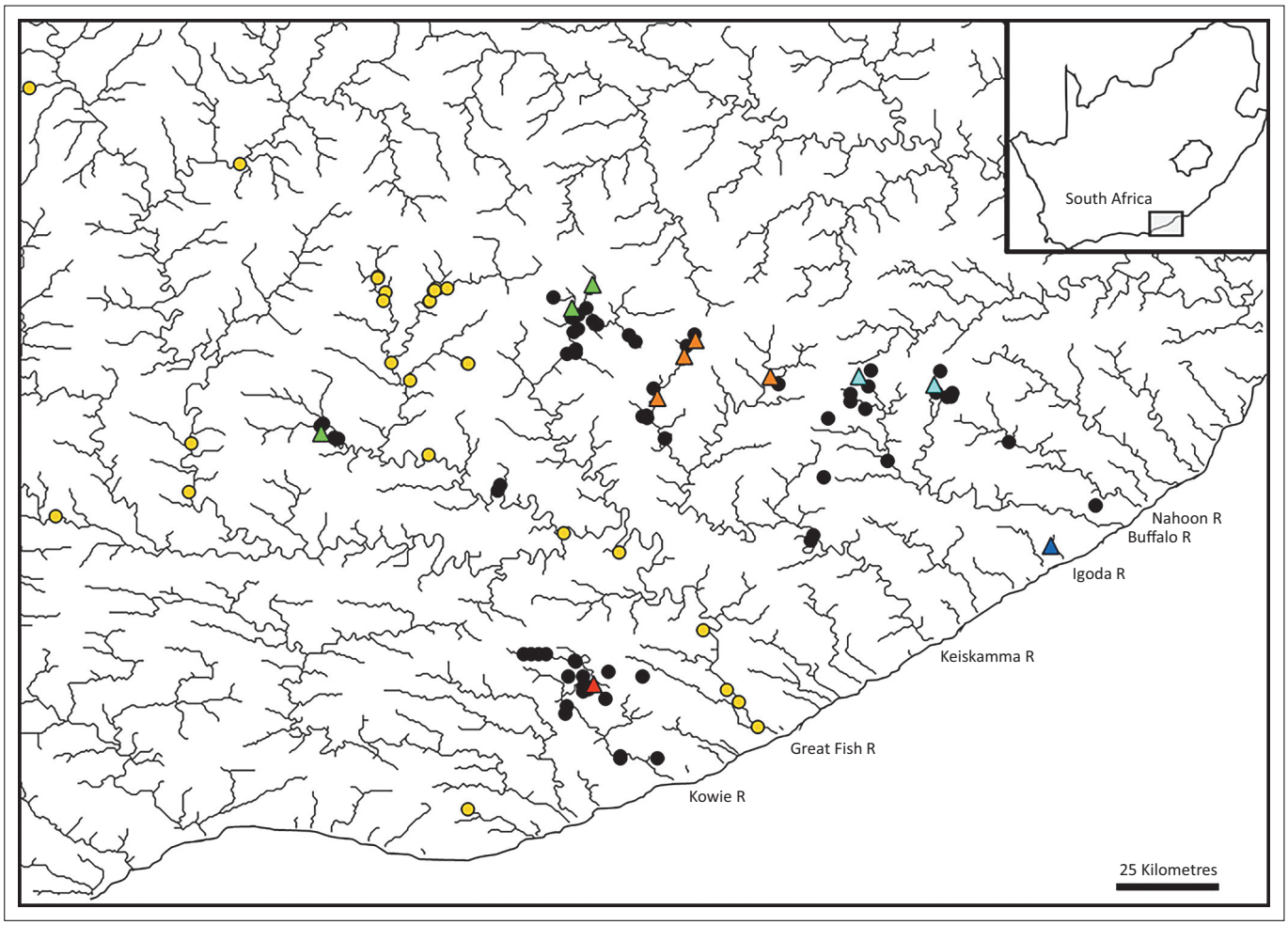
How to cite this article:
Chakona, A., Gouws, G., Kadye, W.T., Mpopetsi, P.P. & Skelton, P.H., 2020, 'Probing hidden diversity to enhance conservation of the endangered narrow-range endemic Eastern Cape rocky, *Sandelia bainsii* (Castelnau 1861)', *Koedoe* 62(1), a1627. https://doi.org/10.4102/koedoe.v62i1.1627

Introduction

Accurate delimitation of species boundaries is a fundamental requirement for formulating environmental policies and spatial conservation planning to prevent loss of biodiversity (e.g. Nel et al. 2011). However, because morphological differences may remain undetected as a result of their subtlelty and the experience or expertise of the observer, there are several cases where two or more morphologically similar species have been mistakenly classified into a single taxon, thus compromising conservation of rare, cryptic and narrow-range species (Bickford et al. 2007). The use of molecular data has resulted in the discovery of new species and several historically isolated lineages within many groups of freshwater fishes that were previously considered to be single wide-ranging species. This is particularly true for the Cape Fold freshwater ecoregion (CFE) in South Africa and the Eastern Zimbabwe Highlands freshwater ecoregion (EZH), where new species, unique lineages and taxonomic conflicts have been discovered in various species groups (e.g. Bronaugh, Swartz & Sidlauskas 2020; Chakona et al. 2018a; Chakona, Swartz & Gouws 2013; Swartz, Skelton & Bloomer 2009; Wishart et al. 2006). Such findings have stimulated renewed interest in the systematics and taxonomic revisions of freshwater fishes in southern Africa (e.g. Chakona & Skelton 2017; Chakona & Swartz 2013; Chakona, Swartz & Skelton 2014; Maake, Gon & Swartz 2014). This information has also been critical in guiding accurate International Union for Conservation of Nature (IUCN) redlist assessments for freshwater fishes in South Africa (Chakona et al. in prep).

In the present study, mitochondrial 16S ribosomal ribonucleic acid (rRNA) sequences were used to explore patterns of spatial genetic structuring in the Eastern Cape rocky, *Sandelia bainsii*, with the aim of illuminating the implications of incomplete systematic knowledge on the conservation of aquatic biodiversity. The Eastern Cape rocky attains the largest size of all anabantid species, reaching a standard length of about 260 mm (Skelton 2001). This species is endemic to South Africa, where it has a restricted distribution, occurring in short sections of the Kowie, Great Fish, Keiskamma, Igoda, Buffalo and Nahoon river systems in the Eastern Cape Province (Skelton 2001; Figure 1). The species is listed by the IUCN as endangered, and the persistence of remnant populations is uncertain as a result of deterioration in water and habitat quality, the spread of non-native piscivores, habitat fragmentation and hydrological modifications (Chakona, Sifundza & Kadye 2018b; Figure 1). Many of the known remnant populations of this species are highly fragmented, and there are concerns that some of these populations may not be viable in the long term (Chakona et al. 2018a).

A previous genetic study based on mitochondrial cytochrome *b* data provided the first evidence for the existence of historically isolated lineages within the Eastern Cape rocky (Roos 2005). This raised concerns that these lineages could be facing a higher risk of extinction as they may potentially have narrower geographic ranges than currently recognised for this species. However, as a result of the small sample sizes and large geographic sampling gaps, this study could not provide a clear determination of the number of lineages and their distribution ranges. The present study builds on the findings of Roos (2005), by collecting samples from all known extant populations of the Eastern Cape rocky to (1) determine the number of unique lineages within this species and (2) provide more accurate distribution ranges of the lineages. We discuss critical future research directions and conservation options to ensure the long-term persistence of the lineages identified within the Eastern Cape rocky.



NRF, National Research Foundation; SAIAB, South African Institute for Aquatic Biodiversity.

FIGURE 1: Historical distribution of the Eastern Cape rocky, *Sandelia bainsii*, based on records from the National Fish Collection at the NRF-SAIAB (black circles), the localities where tissues samples were collected from the Kowie (red triangle), Great Fish (green triangles), Keiskamma (orange triangles), Igoda (turquoise triangle) and Buffalo (navy blue triangles) river systems. The yellow points represent localities where *Sandelia bainsii* was not recorded from recent surveys (2009–2014).

Materials and methods

Sample collection

The present study used data that were collected between 2010 and 2017. The samples were collected from 11 localities in the Kowie, Great Fish, Keiskamma, Igoda and Nahoon river systems, representing all the known remnant populations of *S. bainsii* (Table 1; Figure 1). Sampling was done using fyke nets, seine nets and electric fishing. Because *S. bainsii* is a threatened species, only fin clips were collected for the present study, and all sampled fish were returned to their habitat alive.

Deoxyribonucleic acid extraction, amplification and sequencing

Laboratory work and sequencing were conducted at the Aquatic Genomic Research Platform, NRF-South African Institute for Aquatic Biodiversity (SAIAB). Deoxyribonucleic acid (DNA) was extracted from 21 fin clip samples using the salting-out method (Sunnucks & Hales 1996). A fragment of the mitochondrial 16S rRNA gene was amplified using the primer pair 16Sar and 16Sbr (Palumbi, 1996) and sequenced using the forward primer (16Sar). Each PCR mixture (25 μL) contained 1 × buffer, 2.5 mM $MgCl_2$, 0.8 mM deoxyribonucleotide triphosphates (dNTPs), 0.2 μM of each primer and 0.5 U Taq polymerase and DNA template (final concentration, 4.3 ng/μL – 9.5 ng/μL). The reaction volume was adjusted with dH_2O to a final volume of 25 μL, depending on the volume of the DNA template used. The amplification profile was initial denaturing at 95 °C for 3 min, followed by 35 cycles of denaturing at 95 °C for 50 s, annealing at 50 °C for 30 s and extension at 72 °C for 50 s. The reaction was then completed by a final extension at 72 °C for 10 min. The products were purified using an ExoSAP method (ThermoFisher Scientific). The purified products were then cycle sequenced using ABI (Applied Biosystems, Austin, Texas) Big Dye version 3.1 cycle sequencing. Sequencing was done using an ABI Hitachi 3500 genetic analyser at the NRF-SAIAB.

Data analysis

The sequences were assembled and manually edited to equal lengths using SEQMAN version 7.2.1 (DNA STAR Lasergene Segman Pro) and then aligned in Clustal X2 (Larkin et al. 2007). jModeltest (Darriba et al. 2012) was used to select the best-fit model of nucleotide evolution based on the Akaike information

TABLE 1: Sequence numbers and locality details for the lineages identified within *Sandelia bainsii*.

Date	River system	Latitude	Longitude	SAIAB sequence no.	GenBank accession no.	Lineage name
18/8/2015	Igoda	–33.0736	27.7497	SB1133	MT990986	*Sandelia* sp. '*bainsii* Buffalo'
18/8/2015	Igoda	–33.0736	27.7497	SB1134	MT990987	*Sandelia* sp. '*bainsii* Buffalo'
18/8/2015	Igoda	–33.0736	27.7497	SB1135	MT990988	*Sandelia* sp. '*bainsii* Buffalo'
18/8/2015	Igoda	–33.0736	27.7497	SB1136	MT990989	*Sandelia* sp. '*bainsii* Buffalo'
18/8/2015	Igoda	–33.0736	27.7497	SB1137	MT990990	*Sandelia* sp. '*bainsii* Buffalo'
17/8/2015	Buffalo	–32.6937	27.3188	SB1138	MT990991	*Sandelia* sp. '*bainsii* Buffalo'
19/8/2015	Buffalo	–32.7123	27.4879	SB1139	MT990992	*Sandelia* sp. '*bainsii* Buffalo'
19/8/2015	Buffalo	–32.7123	27.4879	SB1140	MT990993	*Sandelia* sp. '*bainsii* Buffalo'
19/8/2015	Buffalo	–32.7123	27.4879	SB1142	MT990994	*Sandelia* sp. '*bainsii* Buffalo'
19/8/2010	Keiskamma	–32.6943	27.1202	SB1116	MT990985	*Sandelia* sp. '*bainsii* Keiskamma'
19/8/2010	Keiskamma	–32.7418	26.8668	SB1151	MT990995	*Sandelia* sp. '*bainsii* Keiskamma'
19/8/2010	Keiskamma	–32.6943	27.1202	SB1152	MT990996	*Sandelia* sp. '*bainsii* Keiskamma'
19/8/2010	Keiskamma	–32.6121	26.9523	SB1153	MT990997	*Sandelia* sp. '*bainsii* Keiskamma'
19/8/2010	Keiskamma	–32.648	26.9268	SB1154	MT990998	*Sandelia* sp. '*bainsii* Keiskamma'
19/8/2010	Keiskamma	–32.648	26.9268	SB1157	MT990999	*Sandelia* sp. '*bainsii* Keiskamma'
21/2/2014	Kowie	–33.384	26.7236	SB1158	MT991000	*Sandelia* sp. '*bainsii* Kowie'
21/2/2014	Kowie	–33.384	26.7236	SB1159	MT991001	*Sandelia* sp. '*bainsii* Kowie'
21/8/2015	Great Fish	–32.8211	26.1121	SB1187	MT991002	*Sandelia* sp. '*bainsii* Kowie'
21/8/2015	Great Fish	–32.8211	26.1121	SB1188	MT991003	*Sandelia* sp. '*bainsii* Kowie'
21/8/2015	Great Fish	–32.4876	26.7212	SB1190	MT991004	*Sandelia* sp. '*bainsii* Kowie'
21/8/2015	Great Fish	–32.5409	26.6749	SB1191	MT991005	*Sandelia* sp. '*bainsii* Kowie'

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criterion (AIC) as implemented on the CIPRES Science Gateway (Miller, Pfeiffer & Schwartz 2010). A maximum likelihood (ML) tree was generated using RAxML version 8.2.6 (Stamatakis 2014), performed on the CIPRES Science Gateway (Miller et al. 2010). *Sandelia capensis*, *Ctenopoma kingsleyae*, *Ctenopoma patherici* and *Ctenopoma occelatum* were used as outgroups. To further assess the genealogical relationships between sequences among the populations of *S. bainsii*, a haplotype network was generated using the statistical parsimony method implemented in the program TCS (Clement Posada & Crandall 2000). We used PAUP* (Swofford 2002) to estimate the model-corrected genetic distances using the Three Parameter Model (TPM2uf+I) (Kimura, 1981) model of nucleotide evolution.

Results and discussion

The edited alignment of 21 mitochondrial DNA (mtDNA) 16S sequences was 439 bp in length with 13 polymorphic sites that defined four unique haplotypes (Figure 2). The TPM2uf + I (Kimura 1981) was selected as the best model of sequence evolution. The ML tree and the Templeton, Crandall and Sing (TCS) haplotype network revealed strong geographic structuring within *S. bainsii*, with the sequences split into three distinct lineages (Figure 2). These lineages are herein referred to as *Sandelia* sp. '*bainsii* Kowie', which is confined to the Kowie and Great Fish river systems, *Sandelia* sp. '*bainsii* Keiskamma', which is confined to the Keiskamma River system and *Sandelia* sp. '*bainsii* Buffalo', which occurs in the Buffalo and Igoda river systems (Figure 2). These findings are consistent with those of Roos (2005), who identified two lineages corresponding to *Sandelia* sp. '*bainsii* Kowie' and *Sandelia* sp. '*bainsii* Buffalo'. However, Roos' (2005) study did not include samples from the Keiskamma River system. All samples from the Kowie and Great Fish river systems comprised a single haplotype (Figure 2), suggesting that this lineage could have experienced recent range expansion, possibly because of human-mediated translocation, palaeodrainage connections, river captures or intermittent connection of low-drainage divides as inferred for other stream fishes in the genera *Galaxias*, *Pseudobarbus* and *Sandelia* in the CFE (Bronaugh et al. 2020; Chakona et al. 2013; Swartz et al., 2009). Similarly, the sharing of a haplotype between the Buffalo and Igoda river systems also suggests recent connectivity or range expansion between these systems. The existence of these geographically isolated lineages clearly indicates the need for studies that, for example, use multiple genetic markers to determine the patterns of gene flow and investigate the ecology and population dynamics for each lineage. This would provide a better understanding of the mechanisms that have shaped the evolutionary history of *S. bainsii sensu lato*, as well as inform revision of the freshwater fish sanctuaries in South Africa (Nel et al. 2011).

The low haplotype diversity observed in the present study suggests that population decline (Cambray 1996) could have reduced genetic diversity within the Eastern Cape rocky. Advances in molecular techniques that allow extraction and sequencing of DNA from formalin-preserved specimens would provide an opportunity to leverage sequence data from old historical specimens to investigate changes in genetic diversity over time and the possibility of uncovering species or lineages that are now extinct in the wild (Ruane & Austin 2017). The existence of comprehensive samples of *S. bainsii* within the NRF-SAIAB National Fish Collection facility offers a unique opportunity for future studies to investigate the changes in the genetic diversity of the Eastern Cape rocky over time and identify possible causes for those changes.

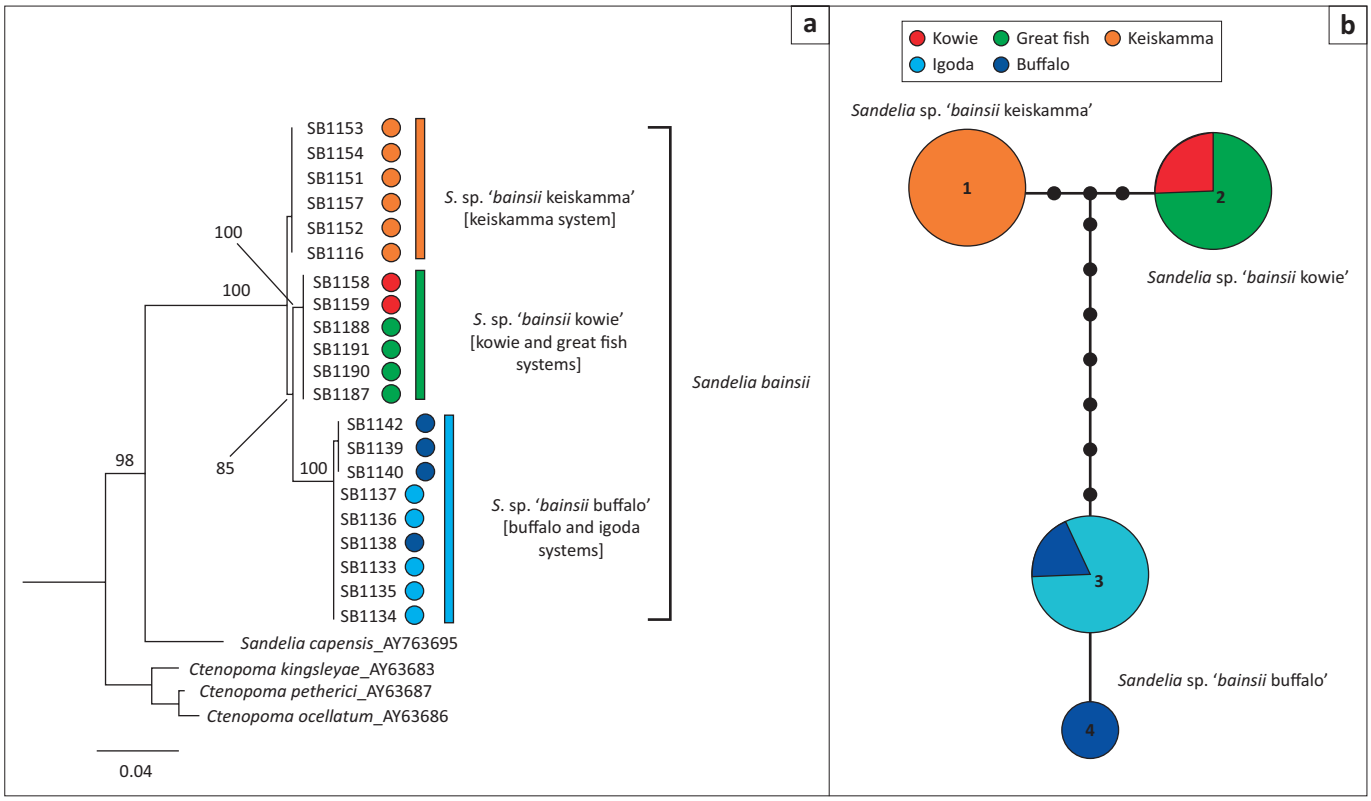

FIGURE 2: Maximum likelihood phylogeny (a) and TCS haplotype network (b) showing the existence of three lineages (*Sandelia* sp. '*bainsii* Kowie', *Sandelia* sp. '*bainsii* Keiskamma' and *Sandelia* sp. '*bainsii* Buffalo') within the Eastern Cape rocky, *Sandelia bainsii*. The river systems where each of these lineages were recorded are indicated.

TABLE 2: Mitochondrial 16S ribosomal ribonucleic acid sequence divergence estimates (%) among the three lineages identified within the Eastern Cape rocky, *Sandelia* sp. '*bainsii* Kowie', *Sandelia* sp. '*bainsii* Keiskamma' and *Sandelia* sp. '*bainsii* Buffalo'

Code	Lineage/species	1	2	3	4	5
1	*Sandelia* sp. '*bainsii* Kowie'	0.0	-	-	-	-
2	*Sandelia* sp. '*bainsii* Keiskamma'	0.95	0.0	-	-	-
3	*Sandelia* sp. '*bainsii* buffalo'	2.42–2.68	2.47–2.73	0.0–0.23	-	-
4	*Ctenopoma petherici*	14.63	14.16	16.58–17.01	-	-
5	*Ctenopoma ocellatum*	11.334	14.54	17.10–17.56	1.18	-
6	*Ctenopoma kingsleye*	13.77	13.34	15.7–16.14	2.66	3.28

Divergences among species of the anabantid genus *Ctenopoma* are presented for comparison.
RNA, ribonucleic acid.

The range of genetic divergence values between *Sandelia* sp. '*bainsii* Kowie' and *Sandelia* sp. '*bainsii* Buffalo' (2.42% – 2.73%) is consistent with that found for interspecific mtDNA sequence divergences in a number of fish genera (Ward 2009) and was also comparable to interspecific differences among some species in the genus *Ctenopoma* (see Table 2), which is the sister genus to *Sandelia* (Rüber, Britz & Zardoya 2006). Our results highlight the need for further studies that integrate genetic (both mitochondrial and nuclear sequences), morphological, osteological, ecological and other biological characteristics (e.g. Puillandre et al. 2012) to determine the taxonomic status of the lineages identified within *S. bainsii*. A recent study by Bronaugh et al. (2019) also revealed the existence of at least three deeply divergent lineages and substantial intralineage genetic and geographic structuring within *S. capensis*, indicating that the current taxonomy obscures the diversity of these anabantid fishes that are endemic to the CFE of South Africa.

Sandelia bainsii was described by Castelnau (1861) based on samples that were collected from the Kowie River system. In the same year, Günther (1861) described another species, *Ctenopoma microlepidotum*, with the type locality details of the holotype vaguely presented as 'freshwaters of the Cape of Good Hope, South Africa'. This species was subsequently synonymised with *S. bainsii* (Skelton 2018). Morphological examination of the types of *S. bainsii* and *C. microlepidotum* is currently ongoing to determine whether they are conspecific and to evaluate whether *Sandelia* sp. '*bainsii* Kowie', *Sandelia* sp. '*bainsii* Keiskamma' and *Sandelia* sp. '*bainsii* Buffalo' constitute distinct taxonomic entities.

The discovery of three genetically distinct and historically isolated lineages within the Eastern Cape rocky, which is already listed as endangered by the IUCN (Chakona et al. 2018b), supports the growing need to expedite discovery and documentation of biodiversity because the existence of hidden diversity in many taxa hampers conservation efforts (e.g. Bickford et al. 2007). As recommended by Moritz (1994), to preserve the evolutionary processes that shaped the genetic patterns within *S. bainsii*, the three lineages need to be

managed separately. This requires close collaboration between researchers, conservationists and local stakeholders (landowners) to develop a sustainable partnership programme that will promote improved catchment management practices for sustainable ecological functioning of the rivers and to protect critical habitats for these lineages.

Previous and ongoing field surveys have reported significant declines in the historical distribution range of *S. bainsii*, with the population in the Kowie River system feared to be nearing extinction, as only two specimens were recorded from comprehensive surveys that were conducted between 2010 and 2017 (Figure 1). The Eastern Cape rocky appears to be highly sensitive to poor water quality, as it has not been recorded from heavily polluted sections of the Kowie and Bloukranz rivers where it was historically abundant. All remnant populations of *S. bainsii* are highly fragmented, because they are isolated by instream impoundments and invasion of the mainstem sections of the rivers by non-native fishes, which may prevent connectivity and hamper gene flow. Field observations suggest that most of these remnant populations are likely to be represented by small population sizes, as they were only recorded in short stretches of the streams where they were found. Empirical data show that population fragmentation leads to rapid deterioration in genetic diversity in small populations because of genetic drift and inbreeding (Bessert & Ortí 2008; Pavlova et al. 2017). As genetic diversity underpins the ability of populations to persist and adapt to environmental changes (Hughes, Schmidt & Finn 2009), there are concerns that the small isolated populations of *Sandelia* sp. '*bainsii* Kowie', *Sandelia* sp. '*bainsii* Keiskamma' and *Sandelia* sp. '*bainsii* Buffalo' could be at high risk of extinction as a result of the possibility of inbreeding depression, loss of fitness and reduced adaptive potential. Future studies should focus on assessing the implications of population fragmentation on genetic diversity and the evolutionary potential of each of these newly identified lineages. In addition, studies should determine effective conservation strategies, such as securing remnant populations to prevent invasion by non-native fishes, rehabilitation of degraded habitats and genetic rescue through assisted gene flow (see Pavlova et al. 2017), where such measures are deemed necessary interventions.

Acknowledgements

The authors hereby acknowledge the use of the equipment provided by the NRF-SAIAB Molecular Genetic Laboratory and the NRF-SAIAB Collection Management Centre. Greg Brett from East London Museum, Leah Sloman, Apelele Zonda and Bosupeng Motshegoa are thanked for their assistance with fieldwork; Leah Sloman for generating the sequences.

Competing interests

The authors have declared that no competing interests exist.

Authors' contributions

This work formed part of P.P.M.'s Hons project; A.C. secured research funding; A.C. and P.H.S. conceived the research; A.C., P.P.M. and W.T.K. conducted the fieldwork; A.C. and G.G. performed the genetic analyses. All authors contributed equally to writing and revising the manuscript.

Ethical consideration

This research was carried out following the evaluation and approval of the sampling protocols by the National Research Foundation – South African Institute for Aquatic Biodiversity Animal Ethics Committee (reference 2014/03). Permits to carry out this research were obtained from the Department of Economic Development, Environmental Affairs and Tourism (Eastern Cape Province) (permit numbers CRO 88/15CR and CRO 44/18CR).

Funding information

This work was supported by the National Research Foundation (NRF) of South Africa under the Foundational Biodiversity Information Programme (FBIP) through Biodiversity Surveys in Priority Inland Areas (IBIP) grant (grant no. IBIP-BS13100251309) through a joint initiative with the Department of Science of Technology, the NRF and the South African National Biodiversity Institute.

Data availability statement

The sequences generated from this study were submitted to GenBank.

Disclaimer

The authors acknowledge that the opinions, findings and conclusions or recommendations expressed in this publication generated by NRF-supported research belong to the authors and that the NRF accepts no liability whatsoever in this regard.

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