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RESEARCH ARTICLE

Population genetics of the endangered Clanwilliam sandfish *Labeo seeberi*: considerations for conservation management

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Abstract

The Cape Fold Ecoregion (CFE) is one of southern Africa's unique aquatic ecoregions and its freshwater fish fauna is characterized by high levels of endemism. As with many other Mediterranean-type ecosystems, the region is also a hotspot for threatened and range-restricted freshwater fish. Many of the CFE's endemic species are at risk for extinction, with declines in population sizes and distribution ranges. The Clanwilliam sandfish *Labeo seeberi* is an example of such a species and is considered one of South Africa's most endangered large migratory cyprinids. This species is endemic to the Olifants/Doring river system in the CFE and has been subject to a major population decline, mainly as a result of invasive alien fish and adverse climate events. Little is known of the genetics of the Clanwilliam sandfish, thus this study aimed to provide basic population genetic parameters to inform future conservation interventions. Both microsatellite and mitochondrial DNA (mtDNA) markers were used to assess populations from three sites within the Olifants/Doring river system. Genetic diversity was moderate to low and did not reflect the drastic decline expected on the basis of previous relative abundance data. This is likely due to a lag effect between ecological/life history demographics (due to juvenile recruitment failures) and population genetic composition. Furthermore, there was limited genetic differentiation between the sampling locations, suggesting a single breeding population, but mtDNA haplotype distribution and slight divergence of the smaller populations does suggest that the population might have become recently fragmented. The results show that the effective population size of the current breeding population might still be sufficient to maintain evolutionary potential in the short term, which could act as a buffer until conservation strategies focusing on protecting breeding animals and maximizing juvenile survival can restore population numbers.

Keywords Cape Fold Ecoregion (CFE) · Freshwater fish · Genetic lag · Microsatellite · mtDNA · Olifant/Doring River

Introduction

Freshwater fishes are some of the most threatened animals globally. This is largely due to fragmentation, degradation, and loss of habitat; water flow modification and pollution; introduction of invasive alien species (as predators and competitors); the increasing threat of climate change; and overfishing (Reid et al. 2013; Dudgeon 2019; Jordaan et al. 2020). The Cape Floristic Region, located primarily within the Western Cape Province of South Africa, is one of the six floral kingdoms of the world and recognized as a global biodiversity hotspot (Myers et al. 2000). The geographical extent of the Cape Floristic Region corresponds to the Cape Fold Ecoregion (CFE), one of the eight aquatic ecoregions of southern Africa (Abell et al. 2008; Chakona et al. 2022). The CFE is moderately diverse in terms of freshwater fish taxa ($n = 40$), but a hotspot for freshwater fish endemism with 92% of these taxa being endemic to the region (Chakona et al. 2022). The number of endemic and threatened taxa continue to increase as a result of ongoing morphological and genetic studies (e.g., Swartz et al. 2007, 2009; Chakona et al. 2013a; Bronaugh et al. 2020) and confirms the suggestion by Linder et al. (2010) that the current taxonomy vastly underestimates the diversity of freshwater fishes of the region. In terms of the International Union for Conservation

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of Nature (IUCN) Red List status, 70% of freshwater fish taxa of the CFE are threatened, with five taxa listed as critically endangered, fourteen as endangered and nine as vulnerable (Chakona et al. 2022).

Alien invasive fish and habitat degradation has been highlighted as the major drivers for the declines in the freshwater fishes in the CFE (Tweddle et al. 2009; De Moor and Day 2013; Weyl et al. 2013, 2014; Chakona et al. 2022). Many populations have become fragmented and are now largely confined to the headwater reaches of streams (Swartz et al. 2004; Tweddle et al. 2009; Chakona et al. 2013b). Conservation management for the persistence and protection of the unique freshwater biodiversity in this region has thus become of increasing importance (Paxton et al. 2012). This is especially important in the case of freshwater fish, as the current protected area network was shown to be largely ineffective for protecting the majority of indigenous freshwater fish species of the region (Jordaan et al. 2020).

The Clanwilliam sandfish *Labeo seeberi* is a large cyprinid species endemic to the Olifants/Doring river system in the CFE. It favors pools or deep runs of larger rivers and migrates upstream into tributary habitat during spawning, which occurs during the austral spring (Paxton et al. 2002, 2012). Hontela and Stacey (1990) reported that the changes in water chemistry, brought on by heavy rain and subsequent flooding of terrestrial vegetation, are the ultimate spawning cue for cyprinids. Potts et al. (2005) reported that flooding has been recognized as the primary factor regulating spawning in the closely related *Labeo umbratus*. Poor rainfall can thus negatively affect spawning cues, causing female fish to retain their eggs until conditions are optimal or reabsorb eggs altogether if conditions do not improve, as was illustrated in *L. umbratus* (Gaigher 1984; Potts et al. 2005).

Labeo seeberi was historically widespread within the Olifants/Doring river system, but currently persists as a fragmented population confined to the middle and northern reaches of the Doring River and its isolated tributaries, namely the Oorlogskloof/Koebee, Gif, Kransgat, Biedouw, Tra-Tra, and Matjies rivers (Paxton et al. 2012; Lubbe et al. 2015). It has been estimated that the species has experienced a more than 90% decline in relative abundance since 2013. This is partly attributed to an estimated 99% decline in annual juvenile recruitment in one of the main spawning areas (Oorlogskloof River) as a consequence of aberrant weather patterns, highlighting the threat of climate change (Cerrilla et al. 2022). The species is currently listed as endangered (Jordaan et al. 2017) and while there is a relatively good understanding of threats to the species, there has not been any study of the current genetic status of the sandfish. This paucity of knowledge may preclude the effective identification and implementation of conservation strategies.

An understanding of the genetic composition of the population will assist in determining conservation units, the extent of genetic diversity and evolutionary potential of the species, and inform captive breeding and release strategies for population recovery and augmentation (Kardos 2021). Riverine environments are often characterized by significant population genetic structuring of aquatic animals due to the system's unique in-stream features (natural and anthropogenic) and hydrodynamics that might act as barriers, restricting animal movement and subsequent gene flow (e.g., Abbas et al. 2010; Peacock et al. 2016; Coleman et al. 2018; Rougemont et al. 2021; Shelley et al. 2022). Using the *COI* mitochondrial DNA (mtDNA) region, Modeel et al. (2023) argued that there was little population genetic structure among *Labeo rohita* (Rohu) populations from south and southeast Asia. However, this mtDNA marker, commonly used as a species identifying barcoding gene due to its low mutation rate, is expected to show high homogeneity within a species (Bhattacharya et al. 2016). Other studies for *L. rohita,* (based on nuclear microsatellite markers and the mtDNA marker, *cyt b*) have presented mixed results, with evidence for limited genetic differentiation and significant genetic divergence between populations depending on the river systems (Luhariya et al. 2012; Sahoo et al. 2014; Behera et al. 2018). Other Indian *Labeo* species, including *Labeo dero* (Chaturvedi et al. 2011) and *Labeo fimbriatus* (Swain et al. 2022) also demonstrated evidence for population genetic stratification. Across the various Asian *Labeo* species, population genetic diversity seems to be wide-ranging depending on the conservation and/or commercial exploitation status of the species.

African *Labeo* species form a distinct clade from the Asian species (Ramoejane 2016; Kebede and Harris 2019), and there are limited studies on population genetic diversity and structure for these species. However, an extensive study using mtDNA and nuclear gene sequences on *L. umbratus*, a closely related species to *L. seeberi*, does suggest that significant population genetic structure could persist within African *Labeo* species on a broad spatial scale (Ramoejane et al. 2021). However, unlike *L. umbratus,*, which has a fairly large distribution range across various river systems (Ramoejane 2016), *L. seeberi* has a restricted distribution within the Olifants/Doring river system. Other cyprinid species within the Olifants/Doring river system seem to exhibit varying degrees of genetic differentiation (Swartz et al. 2004). Hence, the genetic constitution of species, even if restricted to a single river system, might be highly dependent on species-specific life history characteristics and adaptations, breeding behavior, and dispersal ability. The aim of the study was therefore to investigate population genetic parameters, using microsatellite and mtDNA markers, to gain insight into the population dynamics, genetic health, and status of the remaining *L. seeberi* population to inform optimal conservation strategies.

Materials and methods

Sample collection and DNA extraction

The bulk of the sample collection was done by staff from relevant provincial conservation agencies (CapeNature and the Northern Cape Department Nature and Environment Conservation) while conducting routine freshwater fish surveys. Additional samples were collected and donated by staff from the Endangered Wildlife Trust (permit number AAA008-00022). In total, samples were collected from 128 adult fish (> 200 mm) that were sampled from three different localities, two of which were located on the Oorlogskloof River. The upstream site is located within the Oorlogskloof Nature Reserve ($n = 82$, OKNR), while the downstream site is located on Rietkuil Farm at the confluence of the Oorlogskloof and Klein/Kobee rivers ($n = 36$, Riet). These two sites are geographically close, but functionally separated by a waterfall that is a complete barrier to any upstream fish movement. Additional samples were collected from the Bos River ($n = 10$, Bos), a tributary of the Doring River. All samples were taken during a 2013 survey of the greater Doring River system; these sampling locations presented the only sites along the river system where the fish was found during the survey (Fig. 1; Table S1).

Fish were captured using a combination of sein nets and large fyke nets. Fin clips measuring a maximum size of 0.5 cm × 0.5 cm were collected from one of the paired anal fins of each individual and stored in a 2.5 ml tube filled with 100% ethanol. All fish were released back at their site of capture post sample collection. Samples were stored at 4 °C until DNA extractions could be performed using an adjusted cetyl trimethylammonium bromide (CTAB) protocol as described by Justesen et al. 2002.

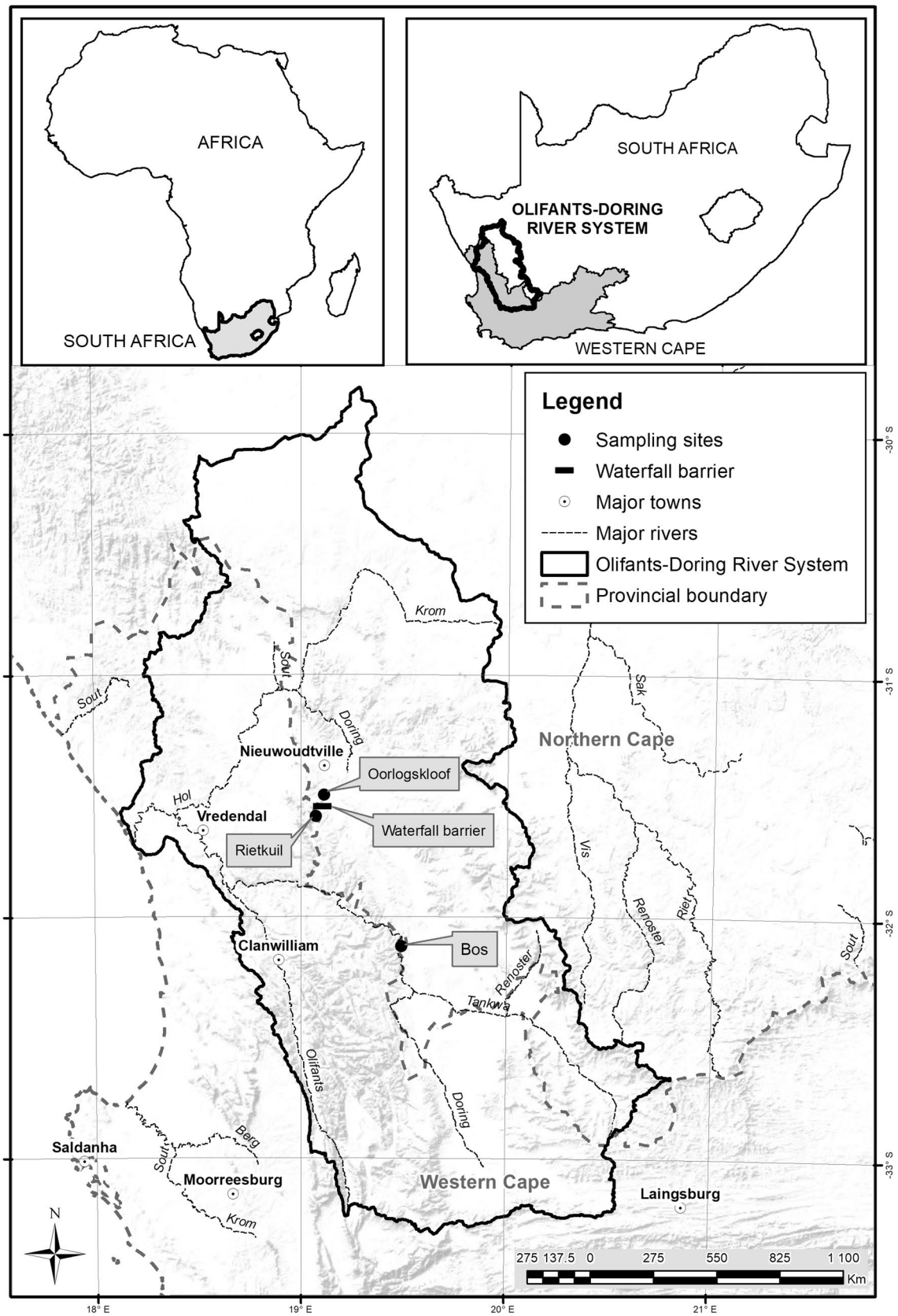
Microsatellite loci and mitochondrial control region amplification

Six microsatellite markers were sourced from literature based on two sister species, *Labeo rohita* (Patel et al. 2011) and *Labeo fimbriatus* (Swain et al. 2012, 2013) (Table S2) and used to genotype the fish from three sampling locations. The polymerase chain reaction (PCR) conditions were as follow: final reaction volume of 10 µl consisted of 1× KAPA Taq Ready Mix (Roche, Basel, Switzerland), 100 ng of DNA and 0.4 µM of each primer. Cycling conditions included an initial denaturation phase at 95 °C for 5 min, followed by 30 cycles of 95 °C for 45 s, 1 min at the annealing temperature (T_A; Table S1), 72 °C for 2 min, with a final extension step at 72 °C for 7 min. Amplified samples were submitted to the Central Analytical Facility (CAF), Stellenbosch University for capillary electrophoresis on a 3730XL Genetic Analyzer (Life Technologies, Carlsbad, California, USA). GeneMapper v4.0 (Life Technologies) was used for binning, allele scoring, and manually curated where needed.

A total of 36 individuals were randomly subsampled to represent the three sampling locations (OKNR—13; Riet—13; Bos—10) for the amplification of the mitochondrial control region (mtCR). A 736 bp fragment of the mtCR was PCR amplified using the primer pair by Lin et al. (2010): Fish G (F): 5′-GCATGGGTCTTGTAATCCGA-3′ and Fish F (R): 5′-TAGTAAGGTCGGGACCATGC-3′, with a total reaction volume of 20 µl Kappa Taq Ready Mix (Roche), using the same reaction composition as for the microsatellite markers. The thermal cycling conditions of these reactions are as follows: an initial denaturation step at 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 s, an annealing temperature of 57 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. PCR products were run on a 1.5% agarose gel at 100 V for 30 min to confirm successful amplification, after which the products were sent to CAF, Stellenbosch University for sample purification using the QIAGEN gel clean-up system and then sequenced using Sanger sequencing chemistry (BigDye® termninator v3.1 cycle sequencing kit, Life Technologies). Sequencing products were purified using Sephadex spin columns (Princeton Separation, Adelphia, NJ) and analyzed via capillary electrophoresis on a 3730XL Genetic Analyzer (Life Technologies). Sequences were aligned using the ClustalW algorithm in MEGA v7 (Kumar et al. 2018), manually edited and trimmed to equal lengths.

Genetic diversity, relatedness, and population demographic analyses

Microsatellite genotypes were evaluated for stuttering, allelic dropout, and the presence of null alleles, with the frequency of null alleles per locus per sampling location calculated with Microchecker v2.2.3 (Van Oosterhout et al. 2004). Genepop on the web v4.2 (Rousset 2008) was used to test for between-loci linkage disequilibrium (LD), within and across sampling locations, as well as for loci deviating from Hardy–Weinberg equilibrium (HWE) expectations (10,000 dememorizations, 100 batches, and 10,000 iterations per batch). Genetic diversity indices were calculated for each sampling location, which included: the average number of alleles per locus (A_N), observed and unbiased expected heterozygosities (H_O and uH_E), Shannon's information index (I), and fixation indices (F_{IS}), as well as standard error (SE) for each mean, in GenAlEx v6.501 (Peakall and Smouse 2012). Polymorphism information content (PIC) was calculated using MSTools v3.0 (Park 2001). The rarefied


AFRICA
SOUTH AFRICA
SOUTH AFRICA
OLIFANTS-DORING
RIVER SYSTEM
WESTERN CAPE
Legend
Sampling sites
Waterfall barrier
Major towns
Major rivers
Olifants-Doring River System
Provincial boundary
Krom
Sout
Doring
Nieuwoudtville
Oorlogskloof
Hol
Vredendal
Waterfall barrier
Rietkuil
Northern Cape
Sak
Vis
Renoster
Riet
Clanwilliam
Bos
Tankwa
Olifants
Saldanha
Berg
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275 137.5 0 275 550 825 1 100
Km
18° E
19° E
20° E
21° E
-30° S
-31° S
-32° S
-33° S

◂**Fig. 1** Map of the Olifants/Doring river system in the Northern and Western Cape of South Africa. The Clanwilliam sandfish is restricted to the northern reaches of the Doring River with recruitment restricted to the Oorlogskloof/Koebee River tributary. Dots indicate sampling locations. The upstream site (above the waterfall barrier) is located within the Oorlogskloof Nature Reserve ($n=82$, OKNR), the downstream Rietkuil site is located at the confluence of the Oorlogskloof and Klein/Kobee rivers ($n=36$, Riet), and the Bos River ($n=10$, Bos) is a tributary of the Doring River

allelic richness per locus (A_R) was estimated using HP-Rare (Kalinowski 2005). The non-parametric Kruskal–Wallis test was performed in XL Statistics v2016.5 (https://software.deakin.edu.au/2017/03/24/xlstatistics) to evaluate the significance ($P<0.05$) of differences in genetic diversity estimates between sampling locations. Mean relatedness per population (i.e., sampling location) was calculated in GenAlEx, using the relatedness estimator, r (Queller and Goodnight 1989). Significance testing by 999 bootstrap replicates for differences between populations was done.

Effective population sizes (N_e) were estimated for each sampling location, using the linkage disequilibrium method as implemented in NeEstimator v2.0.1 (minimum allele frequency of 0.02, assuming a random mating model) (Do et al. 2014), with significance tests set at upper and lower 95% confidence intervals based on bootstrapping. To test for recent bottlenecks, a Wilcoxon signed rank test (Luikart et al. 1998) was performed in Bottleneck v1.2.02 (Piry et al. 1999). Three mutational models were implemented, specifically the infinite allele model (IAM), stepwise mutation model (SMM), and the two-phase model (TPM). Analyses were done using 10,000 replications at the 5% nominal level.

Mitochondrial DNA diversity was determined in DnaSP v5.0 (Librado and Rozas 2009), including the total number of haplotypes (H), hapotype diversity (h), and nucleotide diversity (π) for all sampling locations. The evolutionary relationships among haplotypes was inferred by constructing a median-joining inference network (Bandelt et al. 1999) as implemented in Network v5.0.0.3 (https://www.fluxus-engineering.com).

Genetic structuring analyses

The following microsatellite analyses were performed to assess genetic differentiation between the sampling locations, pairwise F_{ST} estimates, analysis of molecular variance (AMOVA), and multivariate discriminant analysis of principal components (DAPC). Pairwise F_{ST} and an AMOVA were performed (at significance level $P<0.05$) in Arlequin v3.5 (Excoffier and Lischer 2010). Finally, a DAPC plot was constructed using the R packages *ade4* and *adegenet* (Jombart 2008). Prior to running the DAPC, cross-validation was done to determine the optimal number of principal components (PCs) to retain that allowed for the most accurate assignment of individuals (> 80%) to specific genetic clusters. For the mtCR, genetic differentiation among sampling locations was determined in Arlequin, by means of pairwise ϕ_{ST} estimates and AMOVA ($P<0.05$). Additionally, the nucleotide substitution model for the alignment was determined in jModelTest v2.0 (Darriba et al. 2012), with the HKY + G model being the best fit for the dataset. A maximum likelihood (ML) phylogenetic tree was then constructed in MEGA v7 and was bootstrapped with 1000 runs, assuming this substitution model.

Results

Genetic diversity

Microsatellite diversity

A total of 128 individuals were successfully genotyped across six loci, with the average number of alleles ranging from 2 to 23 per maker (Table S3 for detail on per marker diversity across sampling locations). Across all sampling locations, two markers deviated from HWE (Table S3), and no significant linkage disequilibrium was observed. However, locus *Lr_41* displayed evidence for null alleles and deviated from HWE in all populations. Since no significant differences were observed when the locus was excluded from analyses and given the limited number of loci available for analyses, the locus was ultimately retained for all downstream analyses.

Overall, the number of alleles per sampling location was moderate to low (Table 1), ranging from 6.50 (Bos) to 11.17 (OKNR). However, allelic richness was more comparable between the sampling locations (Table 1). Of the three localities, the Bos sample displayed the lowest number of alleles, observed, and unbiased expected heterozygosities (A_N—6.50; H_O—0.583; uH_E—0.043; Table 1). Additionally, the inbreeding coefficients of all three groups were not statistically significantly different from one another, and the low values point to less inbred populations of *L. seeberi*, and none of the populations deviated significantly from HWE on the whole. Low levels of inbreeding were further supported by the mean relatedness (r) estimates that did not deviate significantly from zero for all populations (Fig. 2).

Mitochondrial diversity

A 736 bp fragment of the mtCR was amplified in 37 individuals. Analysis resulted in eleven polymorphic sites, which consisted of ten transitions and one transversion. A total of ten haplotypes were identified, comprised of three high frequency haplotypes (H1, 16.20%; H3, 27.03%; H4, 32.43%; Fig. 3). Notably, haplotype 1 was absent from the Riet

Table 1 Genetic diversity estimates for the Olifants/Doring sampling locations of *Labeo seeberi* based on microsatellite loci and the mitochondrial control region

Sampling location	Nuclear microsatellite loci						mtCR			
	n	A_N	A_R	H_O	uH_E	F_{IS}	*n*	*H*	*h*	π
OKNR	82	11.17	3.35	0.643	0.698	0.052	13	5	0.782 (±0.079)	0.00300 (±0.00036)
Rietkuil	36	9.67	3.40	0.625	0.700	0.062	13	6	0.821 (±0.082)	0.00268 (±0.00045)
Bos	10	6.50	3.40	0.583	0.043	0.043	10	5	0.818 (±0.083)	0.00233 (±0.00049)
Total	128	9.11	3.38	0.617	0.691	0.055	37	10	0.808 (±0.040)	0.00279 (±0.00023)

n sample size, A_N average number of alleles, A_R allelic richness, H_O observed heterozygosity, uH_E unbiased expected heterozygosity, F_{IS} fixation index, *H* number of haplotypes, *h* haplotype diversity, π nucleotide diversity

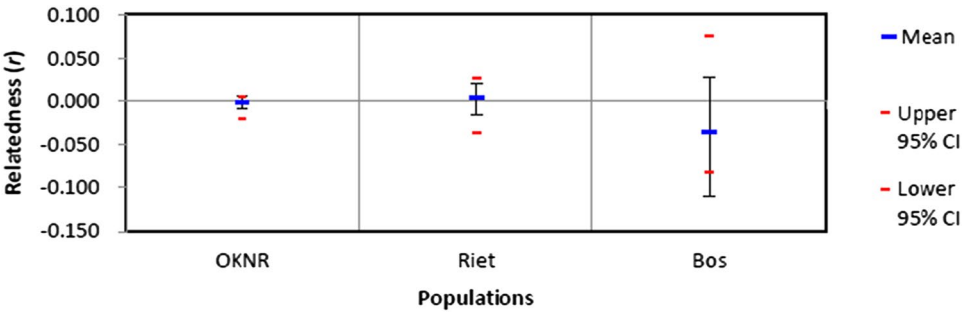

Fig. 2 Estimates of mean relatedness for each of the sampling locations of *Labeo seeberi*. Error bars represent standard error of the mean. Upper (U) and lower (L) 95% confidence intervals for differences among the populations [Oorlogskloof Nature Reserve ($n=82$, OKNR); Rietkuil ($n=36$, Riet); and the Bos River ($n=10$, Bos)]

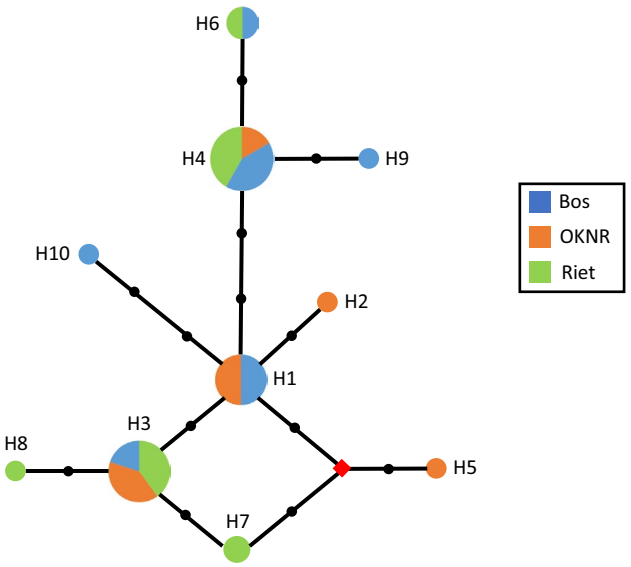

Fig. 3 Median-joining haplotype network of *L. seeberi* mitochondrial control region haplotypes. Haplotypes are separated by black lines, with black dots indicating mutated positions between haplotypes. The size of the haplotype circles is proportional to the number of individuals possessing said haplotype. A total of ten haplotypes were identified, composed of three high frequency haplotypes (H1, 16.20%; H3, 27.03%; H4, 32.43%). The Bos River (Bos; blue, $n=10$); Oorlogskloof Nature Reserve (OKNR; orange, $n=82$); Rietkuil (Riet; green, $n=36$) (color figure online)

Table 2 Pairwise F_{ST} (below diagonal) and pairwise ϕ_{ST} (above diagonal) values between the three sampling locations within the Olifants/Doring river system

	OKNR	Riet	Bos
OKNR		0.022	0.026
Riet	0.007		0.094
Bos	0.002	0.005	

None of the estimates of differentiation were statistically significant

population, while the lower frequency haplotypes appeared to be unique to specific populations. Overall, mitochondrial diversity was relatively high, with haplotype diversity (*h*) and nucleotide diversity (π) ranging from 0.782 to 0.821 and from 0.00233 to 0.00300, respectively (Table 1).

Population differentiation

Nuclear structure

Pairwise F_{ST} estimates were low, ranging from 0.002 to 0.007, with no pairwise comparisons reaching statistical significance at the 1% nominal level (Table 2), indicating little to no genetic differentiation between the sampling locations. Furthermore, the AMOVA (Table 3) supported the lack of genetic differentiation with less than 1% of variation explained by among populations differences (global $F_{ST}=0.005$, $P>0.05$). While the DAPC plot reveals some separation of the Bos population from the OKNR and

Table 3 Analysis of molecular variance (AMOVA) of *Labao seeberi* based on different molecular markers (microsatellite markers and mitochondrial control region, mtCR)

Marker	Source of variation	Variation %	Fixation index
Microsatellite	Among populations	0.548	$F_{ST}=0.005$
	Among individuals within populations	9.197	$F_{IT}=0.097$*
	Within individuals	90.255	$F_{IS}=0.092$*
mtCR	Among populations	4.55	$\phi_{ST}=0.045$
	Within populations	95.45	

*Statistically significant at the 5% nominal level

Rietkuil populations, this is most likely a result of few samples from the Bos area (Fig. 4).

Mitochondrial structure

Pairwise ϕ_{ST} analyses revealed no significant differentiation between the populations, (Table 2), with additional support from the AMOVA results ($\phi_{ST}=0.045$, $P>0.05$; Table 3). The majority of the variance was the diversity of haplotypes within each population contributing 94.45% of the total variance (Table 3). Furthermore, the maximum likelihood tree supports this lack of structure, with no discernible formation of groups within the entirety of the dataset (Fig. 5).

Population demography

The infinite allele model (IAM) showed statistically significant ($P<0.05$; Table 4) evidence of recent population bottlenecks, although this was not supported by the two-phase model (TPM) or the stepwise mutational model (SMM).

Estimations of effective population size (N_e) using the linkage disequilibrium method indicated very low estimates for the lower 95% confidence interval for the Riet and Bos populations, while the OKNR population was higher (Table 4). However, all point estimates using the linkage disequilibrium method were infinite, which is likely a result of using few markers.

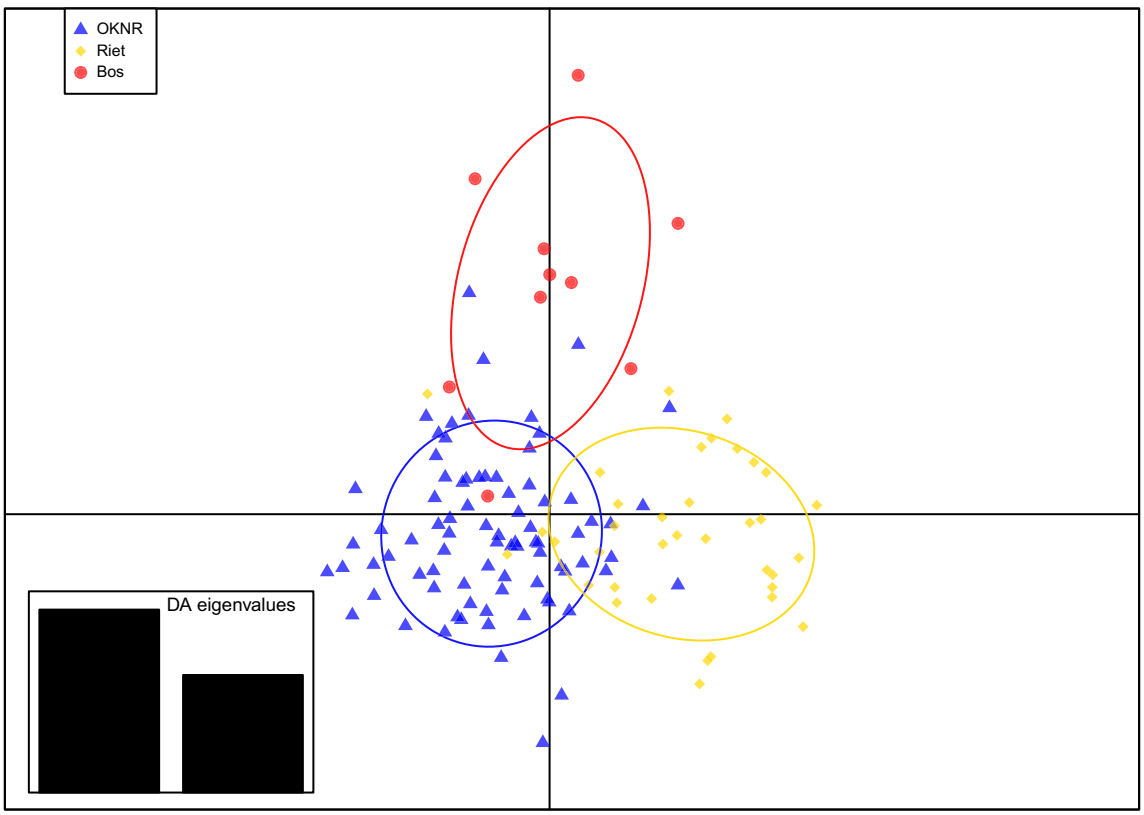

Fig. 4 Multivariate discriminant analysis of principal components (DAPC) plot for *Labeo seeberi* using six microsatellite markers, with sampling locations represented by different symbols and colors [Oorlogskloof Nature Reserve (OKNR; blue triangle, $n=82$); Rietkuil (Riet; yellow diamond, $n=36$); and the Bos River (Bos; red circle, $n=10$)] (color figure online)

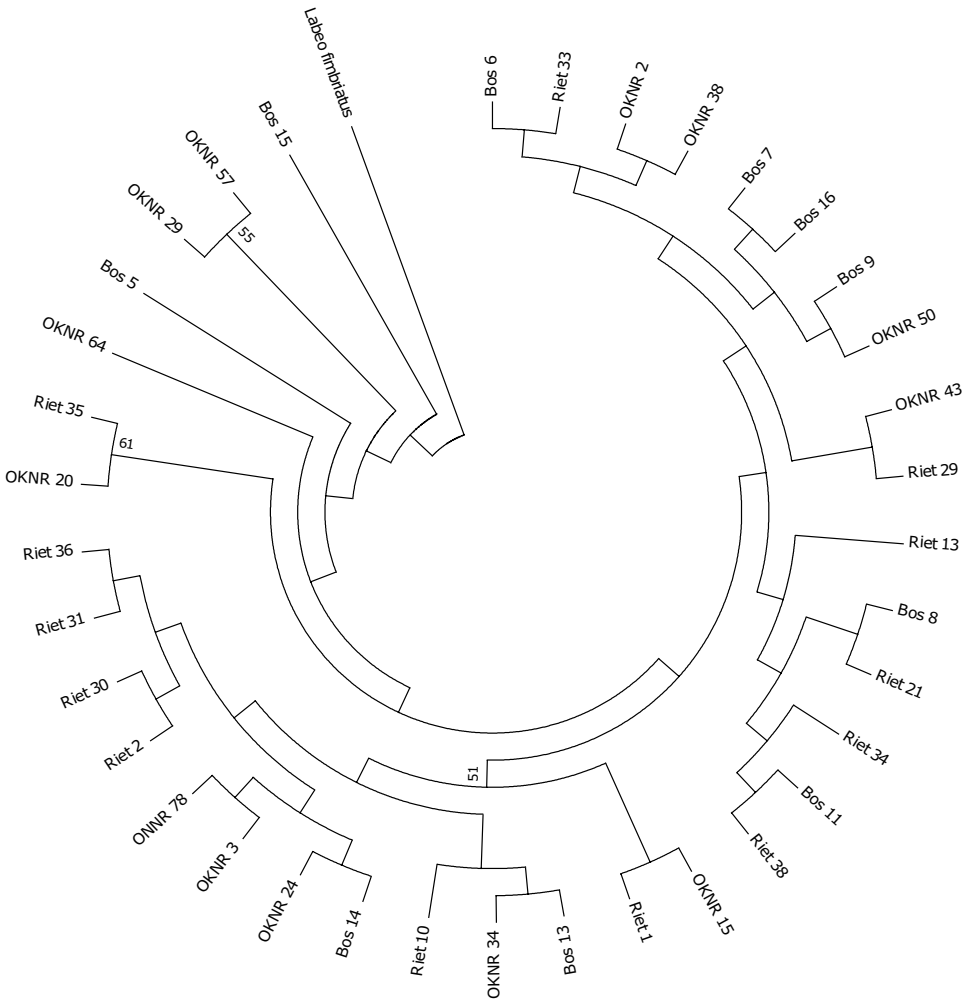

Fig. 5 Maximum likelihood tree of the *Labeo seeberi* populations based on a fragment of the mitochondrial control region. Numbers at nodes indicate bootstrap support for the placement of individuals (only nodes that reached a bootstrap value higher than 50% are indicated), with the tree rooted using a representative control region sequence from a sister species, *Labeo fimbriatus*

Table 4 Bottleneck (Wilcoxon) test under the infinite allele model (IAM), two-phase model (TPM), and stepwise mutation model (SMM), as well as estimates of effective population size (N_e) calculated using the linkage disequilibrium method

Parameter	OKNR	Riet	Bos
Sample size (*n*)	82	36	10
Wilcoxon test			
IAM	< 0.05	< 0.05	*n.s.*
TPM	*n.s.*	*n.s.*	*n.s.*
SMM	*n.s.*	*n.s.*	*n.s.*
N_e	∞ (464.9—∞)	∞ (118.4—∞)	∞ (14.6—∞)

n.s. not significant

Discussion

The cumulative threats present in the CFE have resulted in a decline in both numbers and distribution in almost all of the endemic fish species of the region, including the Olifants/Doring river system (Paxton et al. 2002; Van der Walt et al. 2016; Chakona et al. 2022). These threats include loss of habitat as a result of water overabstraction, water infrastructure such as dams disrupting migration pathways, predatory invasive fish species, and the destruction of riparian zones. This causes fish populations to become fragmented, thereby reducing the population sizes and potentially serving as barriers to gene flow (Paxton et al. 2012; Lubbe et al. 2015; Chakona et al. 2020). Small, isolated populations are intrinsically vulnerable to the loss of genetic diversity through genetic drift and further homogenization of the population through inbreeding (Palstra and Ruzzante 2008; Hare et al. 2011; Chakona et al. 2020). If unchecked, this could impair the evolutionary potential and adaptive capacity of a species, decreasing fitness and leading to eventual extinction (Frankham 2005; Charlesworth and Willis 2009; Hare et al. 2011). This study provides some insight into the population genetics of the disjunct populations of the Clanwilliam sandfish.

Genetic diversity at the microsatellite loci was moderate to low (Table 1) as has previously been reported by Sahoo

et al. (2014) and Singh et al. (2012) for *Labeo rohita* and *Labeo calbasu*, respectively. The number of optimized markers and marker utility evaluation was also similar to Mohindra et al. (2005) who tested microsatellite marker transfer to *Labeo dyocheilus*. This suggested the utility of the cross-species markers that are often criticized for creating ascertainment bias leading to underestimation of genetic parameters in relation to the source species (Barbara et al. 2007). The Bos population did show marginally reduced estimates of diversity, most likely a consequence of the limited sample size, in comparison with the OKNR and Riet populations. However, A_R correcting for unbalanced sampling does suggest that nuclear genetic diversity was similar across sampling locations (Kalinowski 2005). There was a slight homozygous excess for all three sampling locations, with evidence for limited inbreeding (significant F_{IS} and F_{IT} from the AMOVA) but was negligible, as the general population relatedness was practically zero (Fig. 2). This homozygous excess could also be explained by possible null alleles. Alam et al. (2009) and Sahoo et al. (2014) reported very similar diversity estimates for *Labeo rohita* using microsatellite markers. Nonetheless, the genetic data is indicating a population at the initial phases of decline with some loss of genetic diversity (Leberg 2002; Foulley and Ollivier 2006), further supported by evidence for a recent genetic bottleneck under the infinite alleles model (Table 4). This could be attributed to the impacts of invasive black bass (*Micropterus* spp.) that have become established in more than 80% of the river system, with deleterious consequences for native fish biota, resulting in the extirpation of most indigenous fishes from invaded river reaches (Van der Walt et al. 2016). Larger bodied species such as the sandfish persist in these habitats, but at low numbers and in the form of relatively isolated populations. Unusual and erratic climate events, associated with climate change, altering waterscapes, and impacting on fish breeding behavior and juvenile recruitment can also not be excluded (Cerrilla et al. 2022).

Interestingly, estimates of effective population size (N_e) remained high across all three sampling locations. However, the point estimates, based the confidence intervals are imprecise, likely due to the small number of microsatellite markers and limited sample sizes; some caution in the interpretation of the values are thus needed (Waples et al. 2016). This is a common problem for species of conservation concern due to the scarcity and high value of animals (that limits sampling opportunities) and limited genomic resources for an understudied organism that further compounds the estimation of a parameter well known to be difficult to determine and is dependent on various organismal life-history characteristics and demographics (Serbezov et al. 2012). Nonetheless, it has been argued that despite large confidence intervals for estimates of N_e, the LD method remains robust, and that lower confidence bound could still provide a fair assessment for making conservation management judgements (Waples and Do 2010). In their revised recommendations, Frankham et al. (2014) suggested to increase the lower limit for N_e from 50 to 100 in aim of mitigating the immediate effects of inbreeding, and to 500 to ensure at least short-term protection for loss of fitness over the next five generations. Looking at the lower bound N_e estimate for the OKNR population (Table 4), which is considered one of the last major reproducing populations of sandfish, it is already at that 500 cusp. The other two populations seem to have significantly reduced N_e with the Riet population at about the 100 point and the Bos population in critical danger of local extinction with an N_e less than 50. While there is no geographical barrier separating the Riet and the Bos localities, it is likely that the established black bass population in the system has reduced survival of juvenile sandfish to negligible levels, which has effectively fragmented these two populations. Wang et al. (2019) argued that minimum viable population size (MVP) in freshwater fish can be highly variable and that long lived fish with late sexual maturity, long generation intervals, and high fecundity typically require larger MVPs. The authors estimated an upper bound for such species at an MVP of 320 individuals. It has been suggested that MVP should be at least five to ten times greater than the N_e (Frankham 1995; Rosenfeld 2014) and this fits sandfish relative abundance data (Cerrilla et al. 2022). These estimates are also congruent with the patterns of genetic diversity (Baek et al. 2018; Coleman et al. 2018; Martinez et al. 2018).

Cerrilla et al. (2022) documented a significant and drastic decline of more than 92% in the relative abundance of Clanwilliam sandfish in the Oorlogskloof River between 2013 and 2018. This decline was largely driven by a more than 99% decline in young of year individuals, which in turn was attributed to severe weather events including prolonged drought spells. The trend of this severe decline is not reflected in the genetic data, especially for the OKNR population. This is primary because the authors attribute the decline to recruitment failure among the young-of-year, as catch per unit effort for adult fish has remained fairly consistent over the study period. The generation interval is not known for Clanwilliam sandfish, but the closely related *L. umbratus* was reported to reach sexual maturity at 3 and 4 years, respectively, for males and females (Mulder 1973). Estimates based on simulations for other *Labeo* species, *L. niloticus*, *L. roita, and L. coubie*, also suggest that they have fairly long generational times, between 3 and 5 years (www.fishbase.se). Therefore, as genetic analyses are particularly sensitive to changes in the genetic composition of populations over generations (Serbezov et al. 2012), the genetic consequences of the population contraction remain "hidden" if new members are not recruited into the breeding population to form the next generation. This creates a known lag time between ecological and life history demographics

of populations and its corresponding genetic composition and structure (Epps and Keyghobadi 2015). This may also explain the relatively high observed mitochondrial diversity (Table 1) that tend to reflect more historical population dynamics versus the microsatellite data that gives a contemporary analysis (relative to the lag time) (Wan et al. 2004).

Given the critical interplay between N_e, genetic diversity, evolutionary potential, and fitness (Reed and Frankham 2003; Ellegren and Galtier 2016), it is worth noting that there could be considerable differences between short- and long-term N_e, especially for animals with mass spawning and r-selected reproductive strategies (Martinez et al. 2018; Barry et al. 2022). Their reproductive life history characteristics are defined by large variances in breeding success among individuals and mass larval/juvenile mortality rates that leads to reduced N_e in the short-term (Rhode et al. 2017; Martinez et al. 2018; Monteiro et al. 2022). These animals might therefore have some natural resilience to short term larval recruitment failures, as long as the breeding population remains genetically stable and long-lived. Also, high fecundity leads to high mutation rates, that could replenish lost diversity and bolster evolutionary potential relatively quickly, in comparison with animals with k-selected strategies, with effective conservation management (e.g., restoring gene flow, and ensuring equal reproductive success of all breeding animals) and restoration of the natural habitat (Frankham 2015; Ellegren and Galtier 2016; Pavlova et al. 2017; Martinez et al. 2018; Prunier et al. 2023). Given the sampling strategy of this study (only adult fish were sampled) and the stable abundance data for adult fish (from Cerrilla et al. 2022), the current genetic analyses suggest that there is a genetically stable, mature population of Clanwilliam sandfish, with an N_e (approximately 500) that could provide short- to mid-term evolutionary potential and act as a buffer until the natural replenishment of genetic diversity (Martinez et al. 2018). However, it may be concerning that this population is potentially aging, due to the lack of juvenile recruits (Cerrilla et al. 2022). Conservation strategies should thus focus on protecting breeding animals and natural spawning sites and maximizing juvenile survival and broodstock contributions, perhaps through a captive breeding and restocking program or through head-starting initiatives aimed at increasing juvenile survival. Care should, however, be taken to reduce the unintentional impact of hatchery effects leading to genetic erosion (Klütsch et al. 2019; Monteiro et al. 2022).

Neither microsatellite nor mitochondrial data could support sufficient evidence for genetic differentiation within or between the three sampling locations (Tables 2, 3, Fig. 5). However, there was some minor separation of the Riet and Bos population from OKNR and each other, observable on the DAPC plot (Fig. 4). This is likely a sampling artifact of the small populations. But, considering several private mitochondrial haplotypes, and the absence of a high frequency haplotype from the Riet population (H1, Fig. 3), it might suggest that these populations were recently separated. A large waterfall on the Oorlogskloof River serves as a barrier to upstream fish movement, but prior to the establishment of black bass throughout the system juvenile fish from above the waterfall would have migrated downstream toward the mainstream Doring River. At present sandfish do persist downstream of the waterfall but the population is composed almost exclusively of large and old fish. It is thus recommended that all isolated populations of sandfish be managed as a single unit for conservation to maximize population viability and simplify conservation strategies and planning (Funk et al. 2012; Frankham 2015; Pavlova et al. 2017; Coates et al. 2018).

Conclusion

Labeo seeberi is one of the most threatened large cyprinids in southern Africa and is endemic to the Olifants/Doring river system in the CFE. It has been extirpated from much of its historical distribution range and is presently listed as endangered. Contemporary estimates of census population size suggest a more than 90% decline in population numbers in one of the main breeding tributaries, the Oorlogskloof River. Population declines for the greater Doring River system is difficult to quantify given the paucity of monitoring data, but is believed to be severe (Jordaan et al. 2017; Paxton et al. unpublished data). However, the genetic data does not reflect this drastic decline, most likely due to a time-lag between ecological/life history demographics and genetic structure. Nonetheless, the current breeding population, which consists of a single population across three sampling locations, does seem to harbor sufficient genetic diversity to serve as a reservoir for evolutionary potential in the short term (five generations). Conservation strategies must therefore focus on protecting breeding animals and maximizing juvenile survival. Given the high fecundity of the species, there is scope for the "self-restoration" of lost genetic diversity if conservation strategies are successful in securing juvenile recruitment. Conservation interventions that should be implemented with urgency include habitat restoration through management of water abstraction in sensitive catchments and the targeted removal of alien invasive piscivorous fish in priority tributaries where sandfish are known to breed. Removal of undesirable predatory fishes generally require the construction of instream barriers to prevent reinvasion, but this should be implemented within the context of the migratory nature of sandfish, which may prove challenging.

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Author contributions C. Rhode and R. Slabbert conceptualized and designed the study and provided overall project management, coordination, and supervision. S.F. Lesch conducted wet bench work, and preliminary data analysis. K.L. Hull contributed to data analysis and presentation of results. M.S. Jordaan assisted in the coordination of field work and biological sample acquisition. The original draft manuscript was written by C. Rhode and all authors contributed to the reviewing and editing of subsequent versions. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare no competing interests.

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