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Using genetics to prioritise headwater stream fish populations of the Marico barb, *Enteromius motebensis* Steindachner 1894, for conservation action

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South Africa has a relatively large number of threatened freshwater fish species and limited resources to implement conservation programs. *Enteromius motebensis* was regionally prioritised for action because of its conservation status and flagship status in a nationally important aquatic ecosystem. Genetic diversity of *E. motebensis* in headwater refugia of the Groot Marico River Catchment was assessed to determine if genetic diversity is important for conservation planning for this species. The results of the genetic analysis indicate that some prioritisation was possible, with two populations showing evidence of recent isolation.

Conservation implications: We recommend that at least three populations be prioritised for conservation action to ensure maintenance of most of the remaining genetic diversity of the species.

Introduction

Headwater stream ecosystems are often seen as areas of freshwater conservation priority (Abell, Allan & Lehner 2007). The geographical isolation of headwater streams gives rise to high levels of endemism (Abell et al. 2007) and often genetically distinct species (Gomi, Sidle & Richardson 2002). Globally and locally, headwater ecosystems are under increasing threat from human disturbance (Aparicio et al. 2000; Collen et al. 2014; Ellender, Weyl & Swartz 2011; Fausch et al. 2009; Tweddle et al. 2009; Vörösmarty et al. 2010). These threats include, but are not limited to, habitat alteration and water extraction for irrigation purposes and inter-basin transfers, which mediate the introduction of extra-limital and non-native invasive species. Such environmental stressors can permanently alter these systems, driving the extinction of the biota that inhabit them (Fausch et al. 2009; Vörösmarty et al. 2010).

Non-native invasive species pose one of the greatest challenges to conservation in freshwater ecosystems (Dudgeon et al. 2006; Lowe et al. 2000) because they can have a serious impact on native organisms (Moyle & Light 1996; Rahel 2002; Whittier & Kincaid 1999). In South Africa, for example, the largemouth bass *Micropterus salmoides* (Lacepede 1802) was first introduced in 1928 for recreational angling in impoundments (Hargrove et al. 2015). Largemouth bass have subsequently invaded some headwater streams where their predation has altered invertebrate communities (Weyl et al. 2010) and resulted in fragmentation of native fishes such as the Marico barb *Enteromius motebensis* (Steindachner 1894) in the Groot Marico River Catchment, Limpopo River system (Kimberg et al. 2014).

The Groot Marico River Catchment in the North West province (Figure 1) is part of the Limpopo River system and contains several perennial headwater tributaries, which contain unique landscape features and are of significant conservation importance (Nel et al. 2011; Skelton & Ribbink 1994; Smith-Adao et al. 2006). These unique landscape features include groundwater-fed aquifers (dolomitic eyes) that arise from fractures at the contact zones between underlying dolomite intrusions of igneous rock, which provide permanent flow to headwater tributaries (Wellington 1995). In an otherwise arid region, these features support a high number of endemic invertebrate taxa (Grobler, Roux & Koni 2007; Nel et al. 2011). The high level of endemism in conjunction with its free-flowing nature has resulted in the Marico River and its catchment being listed as a National Freshwater Ecosystem Priority Area (NFEPA) (Nel et al. 2011).

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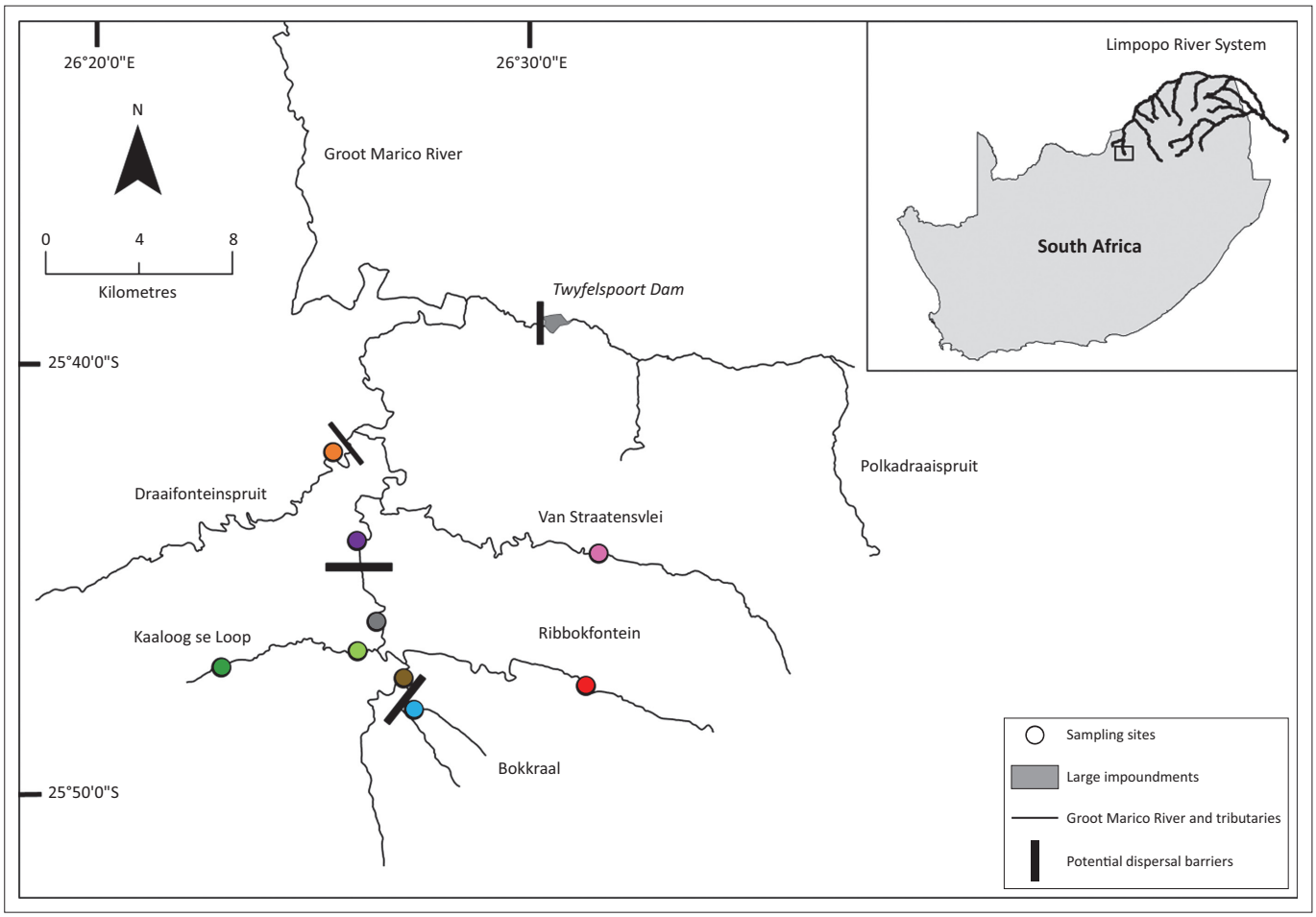


FIGURE 1: Map illustrating the nine sites where *Enteromius motebensis* specimens were collected for genetic analysis, across the upper Groot Marico River Catchment, North West province.

The Marico barb is near-endemic to the Groot Marico River Catchment in the North West province, South Africa, and is considered to be of particular conservation importance because of being Red-Listed as Vulnerable (Engelbrecht & Bills 2007). It is a small (80 mm Standard Length) cyprinid fish characterised by numerous conical tubercles on the snout, forehead and lower jaw and has two pairs of barbels (Skelton 2001). Its reproductive biology suggests that it has an opportunistic life history with high fecundity, an extended breeding season (spring to summer) and early maturity at 50% of their maximum length (Kindler, Wagennar & Weyl 2015). Taxonomically, *E. motebensis* belongs to the Chubbyhead barb group (Engelbrecht 1996). According to Skelton (2001), the other members of this group are *Enteromius anoplus* Weber, 1897 (Chubbyhead barb), *Enteromius gurneyi* Günther, 1868 (redtail barb), *Enteromius amatolicus* Skelton, 1990 (Amatola barb) and *Enteromius breviceps* Trewavas, 1936 (shorthead barb). Previous studies using an allozyme analysis suggest that members of the Chubbyhead barb group are typically restricted to upper catchments of rivers and are often isolated in headwaters of rivers, which results in extensive genetic structuring among populations (Engelbrecht 1996; Engelbrecht & Van der Bank 1994, 1996). In the upper reaches of the Groot Marico River Catchment, *E. motebensis* is limited to the headwater streams of the study area, with a small area of occupancy, which is less than 2000 km^2 (Engelbrecht 1996). This limited distribution may be caused by natural barriers, weirs, dams, water abstraction, habitat alterations and predation by *M. salmoides* (see Smith-Adao et al. 2006). In addition, the fragmentation of this population may further be a result of dispersal ability, habitat preference, isolation by distance and physiological adaptations (Chakona, Swartz & Chakona 2015), all of which result in reduced dispersal success and limited gene flow, which may result in the development of novel species or facilitate localised extinctions (Narum 2006; Segelbacher et al. 2010).

Therefore, an understanding of the genetic diversity and structuring of *E. motebensis* is an important first step towards developing a conservation strategy for this species (Kimberg et al. 2014). The aims of the study were thus: (1) to assess the genetic structure and diversity of *E. motebensis* in the Groot Marico catchment across tributary locations to determine whether *E. motebensis* comprises one population or genetically distinct populations separated by natural barriers, (2) determine whether genetic isolation has occurred, (3) use these data to develop theories on how the ecology of *E. motebensis*, natural landscape and climatic processes may have influenced the genetic structuring of *E. motebensis* populations and (4) identify which populations of

E. motebensis may require a conservation plan in the Groot Marico catchment.

Materials and methods

Study area and sample collection

This study was conducted in the Groot Marico River Catchment in the North West province (Figure 1) part of the Limpopo River system. Samples were collected from the Kaaloog se Loop (henceforth abbreviated to Kaaloog), Draaifonteinspruit, Bokkraal, Ribbokfontein and Vanstraatensvlei tributaries as well as from the Groot Marico main stem (Figure 1). There are five known potential in-stream barriers (Roux 2010) that may restrict upstream migration (Hughes, Schmidt & Finn 2009). Four of these barriers are natural in the form of waterfalls and are found in the Draaifonteinspruit, Bokkraal and Polkadraaispruit tributaries and in the upper reaches of the Groot Marico main stem. The last barrier is an artificial weir on the lower reaches of the Draaifonteinspruit tributary (Figure 1).

Sixty-eight *E. motebensis* individuals were collected from nine locations (Figure 1, Table 1) in March and November 2012 using a SAMUS backpack electrofisher (90-Hz pulsed-DC current) and seine netting. At each locality, up to 10 individuals of *E. motebensis* were euthanised with clove oil (0.2 mL/L). The rest of the captured fish were returned alive. Small pieces of muscle tissue from each specimen were then removed using a dissection blade and placed into 1.5-mL vials containing 98% ethanol. The remaining specimens served as voucher specimens and were fixed in 10% formalin and later transferred to 70% ethanol. Voucher specimens were accessioned into the National Collection of the South African Institute for Aquatic Biodiversity (SAIAB) in Grahamstown, South Africa. The tissue vials were then stored in a freezer at -20 °C for DNA extraction, and once all laboratory work was completed, the tissue vials were transferred to SAIAB's Biomaterial Bank for long-term storage in ultra-deep freezers at -70 °C.

DNA extraction, amplification and sequencing

Total genomic DNA was isolated from muscle tissue using the nucleic acid and protein purification extraction kit, Nucleospin Tissue (Machery-Nagel, Germany). A 947-base pair fragment of the mitochondrial ND2 gene was amplified with the primers ND2-F and ND2-R (Kocher et al. 1995). The polymerase chain reactions (PCRs) were performed with a Veriti® 96-well thermal cycler (Applied Biosystems, North America). Each reaction mixture (25 µL) contained 1x reaction buffer, 2.5 mM $MgCl_2$, 0.8 mM of each dNTP (Kapa Biosystems [Pty] Ltd, Cape Town), 0.2 mM of each primer, 1 U Super-Therm Taq polymerase (Southern Cross Biotechnology, Cape Town), and 100 ng – 200 ng of template DNA. The PCR profile for amplification for the mitochondrial ND2 gene was 95 °C for 3 min, followed by 35 cycles of (1) 95 °C for 45 s, (2) 50 °C for 45 s and (3) 72 °C for 1 min, and then a final extension at 72 °C for 5 min. The ND2 gene region of 68 individuals was sequenced with the forward primer only, as no ambiguities or sequencing errors were detected. Automated sequencing was performed at Macrogen Inc. (South Korea) using an ABI 3730xl DNA Analyser (Applied Biosystems).

TABLE 1: Localities and sample sizes (*n*) analysed for the mitochondrial nucleotide diversity 2 gene of *Enteromius motebensis*.

Site	Locality	Locality code	ND2 analysis (*n*)	HD (*h*)	ND (π)
1	Lower Draaifonteinspruit	LD	7	0.667	0.001
2	Upper Kaaloog	UK	2	1.000	0.004
3	Lower Kaaloog	LK	7	0.810	0.002
4	Above Bokkraal waterfall	ABW	11	0.439	0.002
5	Below Bokkraal waterfall	BBW	12	0.727	0.001
6	Upper Ribbokfontein	UR	12	0.742	0.005
7	Middle Vanstraatensvlei	MV	4	0.833	0.001
8	Groot Marico Sonop	GMS	5	0.893	0.009
9	Below Groot Marico waterfall	BGMW	8	0.900	0.005
Total	-	-	**68**	**0.883**	**0.007**

ND, nucleotide diversity; HD, haplotype diversity.

Statistical analysis

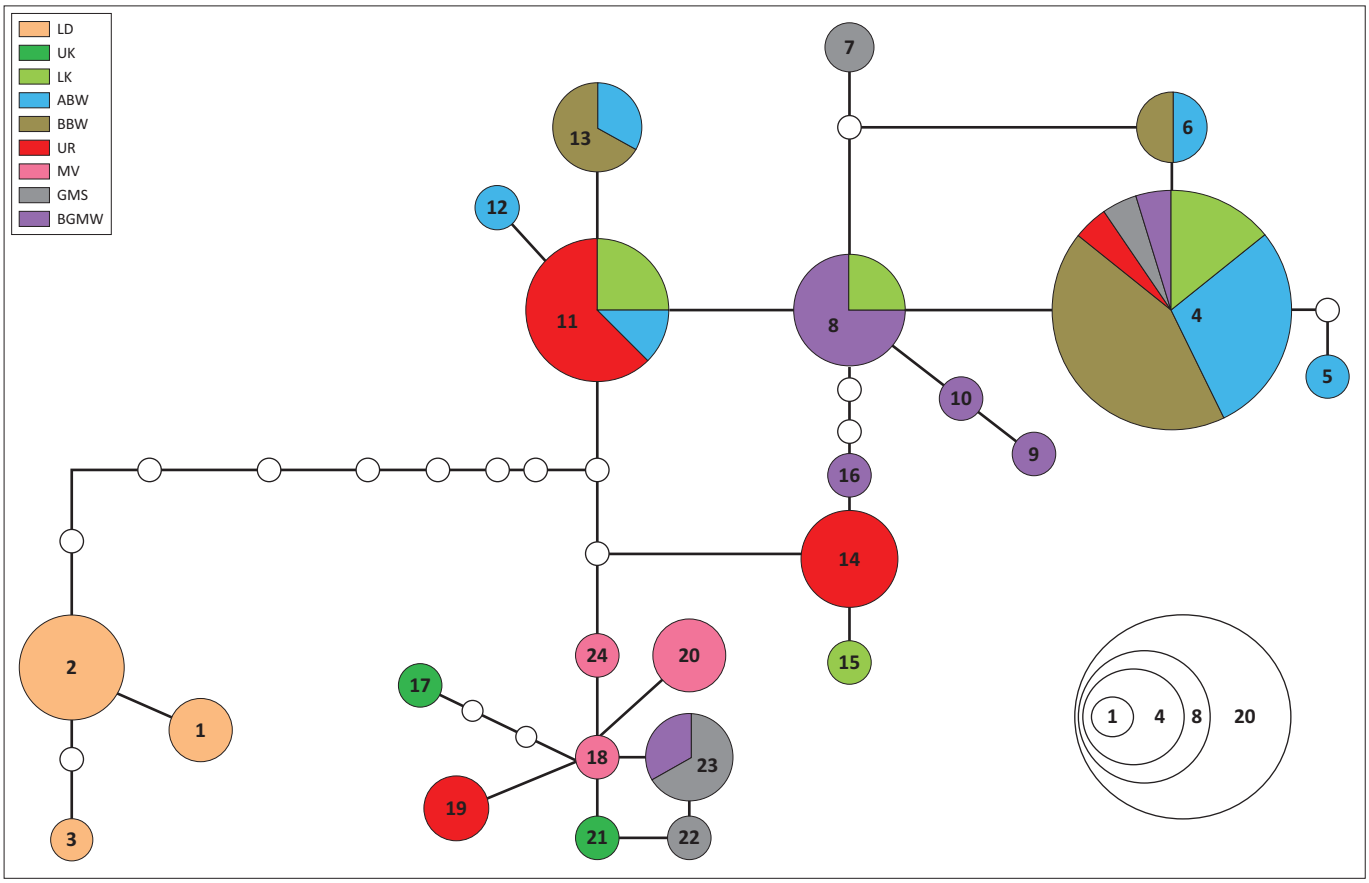
Sequences were cleaned and trimmed manually to equal lengths using the program SeqMan 10.2.1 (DNASTAR, Madison, WI, USA) and were aligned using the program ClustalX (Larkin et al. 2007). Nucleotide sequences of the protein-coding ND2 gene were translated to amino acid sequences to check for stop codons and errors using the program DnaSP 5.10 (Librado & Rozas 2009). All sequences were submitted to GenBank (accession numbers: KT070326–KT070393). Unique haplotypes were identified using DnaSP 5.10 (Librado & Rozas 2009). Haplotype diversity (*h*) and nucleotide diversity (π) were calculated for each location and the overall locations using ARLEQUIN ver. 2.000 (Schneider, Roessli & Excoffier 2000) (Table 1). Parsimony networks were constructed using the program TCS 1.2.1 (Clement, Posada & Crandall 2000).The best fitting model of molecular evolution for the ND2 data was estimated using Akaike's Information Criterion (Burnham & Anderson 2002) as implemented in jModelTest 2 (Darriba et al. 2012) and pairwise Φ_{ST} values were calculated in ARLEQUIN ver. 2.000 (Schneider et al. 2000) to determine the degree of genetic differentiation among locations. Model-corrected distances between unique haplotypes were calculated using PAUP* (Swofford 2002).

Results

For the mitochondrial ND2 gene, TRN+G (Tamura & Nei 1993) was the substitution model that best fit the data, with a gamma value of 0.128. Analysis of 68 *E. motebensis* individuals from nine locations resulted in 24 haplotypes defined by 38 variable sites. The overall nucleotide diversity for all tributary locations was low (π = 0.007), with the Vanstraatensvlei, Lower Draaifonteinspruit and Below Bokkraal Waterfall tributary locations having the lowest nucleotide diversity (π = 0.001) (Table 1). Overall haplotype diversity was high (H_D = 0.883), and haplotype diversities at each of the locations ranged from 0.439 (Bokkraal tributary) to 1.00 (Upper Kaaloog tributary) (Table 1).

The parsimony network revealed that the Draaifonteinspruit tributary did not share any of its haplotypes (1, 2 and 3) with other tributary locations (Figure 2). There was also minor differentiation among the remaining tributary locations that could indicate disrupted gene flow patterns (Figure 2). For example, the Vanstraatensvlei tributary did not share any of its haplotypes (18, 20 and 24) with other tributary locations. The Kaaloog, Bokkraal, Ribbokfontein tributaries and the Groot Marico main stem shared at least a proportion of the remaining haplotypes (Figure 2). Sequence divergence in the Draaifonteinspruit tributary ($0\% < D < 0.3\%$) and the Vanstraatensvlei tributary ($0\% < D < 0.2\%$) was relatively low compared to the range of genetic divergence ($0\% < D < 2.43\%$) among the remaining 18 haplotypes (Table 2).

Significant high values were observed in all analyses of genetic structure because of the lack of sharing of haplotypes between Draaifonteinspruit and the other tributary locations. However, because of the small sample size, the full extent of diversity at each location may not have been captured. High Φ_{ST} values (> 0.600) were found between the Upper Kaaloog and other tributary localities (Table 2). A similar pattern ($\Phi_{ST} > 0.600$) was found between the Middle Vanstraatensvlei and six other localities (Table 2). Low Φ_{ST} values (< 0.200) among some of the locations suggest relatively recent (e.g.



Small open white circles that are not numbered indicate missing haplotypes. Each line in the network represents one mutational change. The Draaifonteinspruit (orange) and Vanstraatensvlei (pink) tributaries did not share any of their respective haplotypes (haplotypes 1, 2 and 3 for Draaifonteinspruit and haplotypes 18, 20 and 24 for Vanstraatensvlei) with other tributary locations.

FIGURE 2: Parsimony network with 95% plausible set of mitochondrial ND2 allele connections (numbered circles) constructed with the program TCS 1.2.1 (Clement et al. 2000) for *Enteromius motebensis* (see Table 1 for location key codes). The size of the circles indicate the relative frequency of haplotypes (total $N = 68$) (see Table 1).

TABLE 2: Ranges of genetic divergences (%) (above the diagonal), Φ_{ST} values (below the diagonal) for pairwise comparisons for *Enteromius motebensis* from nine populations in the Upper Groot Marico River based on nucleotide diversity two sequences.

Site	1 LD	2 UK	3 LK	4 ABW	5 BBW	6 UR	7 MV	8 GMS	9 BGMW
LD	0.10–0.30	1.71–2.35	1.06–1.76	1.18–1.88	1.19–1.74	1.17–2.04	1.45–2.02	1.45–2.16	1.18–2.02
UK	0.92*	0.00–0.44	1.55–2.29	1.68–2.43	1.69–2.26	0.44–1.96	0.10–0.44	0.55–2.27	0.21–2.27
LK	0.85*	0.85*	0.00–0.57	0.10–0.91	0.10–0.93	0.22–1.99	1.29–1.97	0.32–2.10	0.10–1.97
ABW	0.89*	0.89*	0.01	0.22–0.55	0.10–0.55	0.43–2.12	1.42–2.10	0.32–1.96	0.10–1.83
BBW	0.92*	0.93*	0.11	-0.05	0.00–0.44	0.44–1.96	1.43–1.94	0.22–1.82	0.21–1.69
UR	0.67*	0.61*	0.09	0.27*	0.34*	0.00–1.67	0.21–1.65	0.32–1.98	0.10–1.97
MV	0.93*	0.25	0.87*	0.90*	0.94*	0.63*	0.11–0.21	0.21–1.95	0.10–1.95
GMS	0.70*	0.15	0.45*	0.55*	0.60*	0.29*	0.27	0.00–1.83	0.10–1.82
BGMW	0.76*	0.71*	-0.01	0.13*	0.19*	0.06	0.74*	0.25	0.10–1.69

*, $p < 0.05$

before bass introduction) or potentially ongoing gene flow (Table 2).

Discussion

Enteromius motebensis is a key component of the unique headwater stream biota that characterises the Groot Marico NFEPA, being near-endemic and restricted to the near-pristine headwaters of this vulnerable catchment (Engelbrecht & Bills 2007; Nel et al. 2011). It is directly threatened by invasive largemouth bass *M. salmoides* (Kimberg et al. 2014) and is likely to require conservation action to avoid a decline in its conservation status from Vulnerable to Endangered. To aid such future efforts, molecular analyses demonstrated the presence of significant genetic structuring among the sampled populations of *E. motebensis* and consequently identified which populations require conservation prioritisation within the Groot Marico catchment.

The current study demonstrates that *E. motebensis* locations in the upper Groot Marico River Catchment show relatively recent isolation of at least one location, the Draaifonteinspruit tributary, and significant genetic structuring and diversity among some of the remaining locations (Vanstraatensvlei tributary). This genetic structuring may be a result of a number of factors such as in-stream barriers, habitat preferences, tributary flow regimes or isolation by distance. Furthermore, results indicated that the overall haplotype diversity across locations of *E. motebensis* was high and haplotype diversities at each location for *E. motebensis* was relatively high.

In the upper Groot Marico River Catchment, the lack of gene flow from the Draaifonteinspruit tributary to the remaining populations is potentially the result of a natural waterfall barrier below the confluences of the Kaaloog and Ribbokfontein tributaries (Figure 1). This barrier may interrupt upstream dispersal of *E. motebensis* from Draaifonteinspruit. However, the apparent lack or limited downstream migration from upstream areas to the Draaifonteinspruit and Vanstraatensvlei tributaries cannot be explained by natural barriers because *E. motebensis* should be able to move downstream over such obstacles. In addition, the waterfall is only approximately 1 m high. Therefore, it is possible that this barrier may be breached during periods of flooding, which would allow upstream migration. While the artificial weir on the Draaifonteinspruit can prevent present-day upstream migration of *E. motebensis* or invasion by *M. salmoides*, the genetic results are likely a result of more historic processes (vicariance, habitat fragmentation, and drainage alterations etc., Hughes et al. 2009). Therefore, the high level of genetic structuring between these areas is surprising.

Enteromius motebensis inhabits shallow slow-flowing pools in small streams (Engelbrecht & Bills 2007), making it a rhithronic pool specialist typically associated with headwaters of mountain streams. Therefore, this habitat preference may limit downstream migration of *E. motebensis*, a supposition that is supported by Kimberg et al. (2014), who found that *E. motebensis* was most abundant in the upper reaches of the catchment and seldom penetrated into the lower stream reaches where tributary confluences are located. Moreover, Ellender, Woodford and Weyl (2015) demonstrated that the Eastern Cape redfin *Pseudobarbus afer* (Peters 1864) resisted displacement during a high-magnitude flood in a headwater stream in the Eastern Cape. Therefore, the possibility that *E. motebensis* may display similar resistance to downstream movement cannot be excluded as a potential mechanism for restricted gene flow.

The Vanstraatensvlei and Draaifonteinspruit tributaries have a lower flow volume and lower gradient compared to other tributaries in the Groot Marico River Catchment (Roux 2010), which may have contributed to disruption of gene flow. The Draaifonteinspruit, in particular, has a weak seasonal base flow because of lower dependence on dolomitic groundwater, resulting in naturally intermittent flow near its confluence with the Marico River (Roux 2010). Thus, naturally intermittent connectivity between Draaifonteinspruit and the other tributary locations may also have contributed to isolation.

It is also possible that isolation by distance played a role in isolating the Draaifonteinspruit and Vanstraatensvlei populations, and a further investigation to formally test this hypothesis should be conducted. Isolation by distance occurs when organisms have limited dispersal ability, which increases genetic differentiation between sites with increasing geographic distance between them (Wright 1943). Slatkin (1993) further suggested that in highly connected populations, isolation by distance will occur if dispersal distance of an individual is less than the range of the species. Therefore, it may be possible that *E. motebensis* individuals have small home ranges or very strong preference for tributary habitats and are able to maintain their position in the tributaries during flooding periods.

Conclusion

With limited resources, the maintenance of the genetic structuring and high genetic diversity of *E. motebensis* can be achieved by prioritising a subset of populations. At least three populations from the genetic evidence are necessary to represent most of the detected genetic diversity of the species. However, in order to validate this finding, more than one genetic marker needs to be analysed (nuclear markers and microsatellites), sample sizes of *E. motebensis* need to be increased and specific adaptations of *E. motebensis* need to be studied.

From the results of this study, it can be seen that the Draaifonteinspruit and Vanstraatensvlei tributaries have to be secured for *E. motebensis* because they have unique haplotypes that do not occur elsewhere. This was also evident in populations of the stargazer mountain catfish *Amphilius uranoscopus* (Pfeffer 1889) that also occurs in the Groot Marico catchment (KA Van der Walt, unpublished data). Therefore,

among the remaining populations, the Kaaloog tributary has the highest genetic diversity of all the *E. motebensis* populations and will contribute most towards maintaining the detected genetic diversity.

Micropterus salmoides is present in the dolomitic eye of the Kaaloog tributary, which prevents this area from being an effective refuge area for *E. motebensis*. Therefore, removal of these non-native invasive fish, together with local stakeholder education to prevent their re-introduction is the single highest priority conservation action. Furthermore, non-native invasive fish management and reduction of other threats to the Groot Marico River Catchment will directly and indirectly help to improve the conservation status of *E. motebensis* in future, should more resources become available.

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Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors' contributions

This article is based on the Master's thesis of K-A.v.d.W. K-A.v.d.W. was responsible for sample collection, sample processing, laboratory analysis, genetic analysis, interpretation of data and writing the article. E.R.S. played a co-supervisor role. Assisted conceptualisation and planning of the research, assisted with genetic analysis, interpretation of data and writing the article. D.W. assisted with sample collection, construction of map, interpretation of data and writing the article. O.W. conceptualised the overall research programme, sourced funding, planned field research, assisted with sample collection, interpretation of data and writing the article.

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