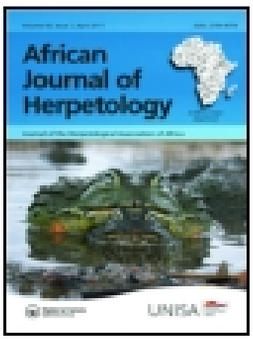

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Lack of phylogeographic structure in the endangered Pickersgill's Reed Frog; *Hyperolius pickersgilli* (Raw, 1982)

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ABSTRACT

The Endangered Pickersgill's Reed Frog (*Hyperolius pickersgilli*) is endemic to South Africa and restricted to the KwaZulu-Natal (KZN) coast. The natural habitat of *H. pickersgilli* is limited to fragmented patches of coastal reed-bed wetland, the majority of which continues to undergo transformation and degradation caused by urbanisation, agriculture, mining and forestry. These changes have resulted in the steady reduction of suitable, quality habitat and severe fragmentation. In the current study we employed mitochondrial DNA and species-specific microsatellites markers (developed in the current study) to investigate the genetic structure and diversity of *H. pickersgilli*. Genetic markers revealed moderate to high levels of genetic diversity throughout the remnant groups and absence of specific phylogeographic structure among individuals sampled across twelve localities throughout the range of the species. Results from the current study indicate that gene flow between *H. pickersgilli* individuals is not restricted, whereby neighbouring groups may interact with each other through continued migration, thereby facilitating possible range expansion should habitat be available. However, the need for continued conservation of the *H. pickersgilli* population through the protection and management of its natural habitats should remain a top priority in order to conserve representative levels of genetic diversity.

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Introduction

Habitat loss and fragmentation caused by human activities change natural habitats into increasingly anthropogenic landscapes. These processes can indirectly or directly affect the genetic structure of wild populations, because of a restriction of gene flow or because of increasing levels of genetic drift and inbreeding (Reed & Frankham 2003).

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Inbreeding depression can lead to a reduction in fitness whereby individuals have a reduced ability to cope with a changing environment, reduced survival and reproduction, as well as extinction of local sub-populations (Frankham 1995; Mech & Hallett 2001; Reed & Frankham 2003). Amphibians are often described as being highly sensitive to habitat fragmentation (Hels & Buchwald 2001; Gibbs & Shriver 2005; Noël et al. 2007) and loss of habitat and population isolation have been identified as two of the main causes of amphibian declines (Cushman 2006; Gardner et al. 2007; Hamer & McDonnell 2008).

In the current study, we present a genetic analysis on Pickersgill's Reed Frog (*Hyperolius pickersgilli*; Raw, 1982; Fig. 1), which inhabits coastal wetland across a fragmented range along the South African east coast that has been transformed and degraded, because of various anthropogenic activities. The species was discovered in the late 1970s and described by Raw (1982). Species accounts can also be found in Lambiris (1989), Passmore & Carruthers (1995), Schiøtz (1999), Channing (2001), Bishop (2004), Du Preez & Carruthers (2009) and Measey (2011). *Hyperolius pickersgilli* is endemic to the KwaZulu-Natal coast (<380 m above sea level) from Sezela in the south, through Durban, Mtunzini, Richards Bay and to St Lucia (iSimangaliso Wetland Park) in the north (Raw, 1982; Tarrant & Armstrong 2013; Fig. 2). The species is highly habitat specific in terms of its ecology, occurring within 15 km of the coastline and requiring the unique vegetation structure found in Indian Ocean Coastal Belt Wetland (itself of a threatened status). The species inhabits reed-bed wetland systems and breeds in permanent standing water, at depths between 20 and 80 cm (Tarrant & Armstrong 2013). Alexander (1990) described the known localities of *H. pickersgilli*, whereas Tarrant & Armstrong (2013) used predictive modelling and surveying of high probability areas (>60%) to examine the potential distribution of the species based on known localities. Findings based on MaxEnt modelling indicate that the extent of occurrence (EOO) was more extensive than previously thought but that actual area of occupancy (AOO) was reduced. The most recent Red Listing assessment puts the EOO at 4 768 km^2 and the AOO at 12 km^2 (IUCN 2016). The total number of known sites for the species is approximately 25 as of January 2018. Of the 25 known population localities, only two are found in Protected Areas (Umlalazi Nature Reserve and iSimangaliso Wetland Park). The declining AOO, and increased perceived threats, have resulted in the IUCN Red List classification of *H. pickersgilli* being updated from Rare (1994) to Vulnerable (1996), Endangered (2004), Critically Endangered (2010) and most recently back to Endangered (2016). The reclassification to Endangered stemmed from an increase in known localities, as a result of ongoing surveys.

Figure 1. Representative images of Pickersgill's Reed Frog (A) Male and (B) Female. Photo credit Nick Evans.

Habitat loss has been identified as a major threat to *H. pickersgilli* populations (Tarrant & Armstrong 2017). As a result of urbanisation, mining, agriculture (in particular drainage of wetlands for the establishment of sugar cane) and sylviculture (especially eucalyptus plantations, which can dry out wetlands) has caused several historically known sites to be eliminated, whereas many others are experiencing a decline in the quality of their habitat (Johnson & Raw 1989; Armstrong 2001; Bishop 2004; Measey 2011; Tarrant & Armstrong 2017). This transformation has resulted in the steady reduction of suitable habitat and the severe fragmentation of this species' distribution whereby the natural habitat of *H. pickersgilli* is often restricted to small patches. Lack of management and minimal formal protection puts these sites at risk of further deterioration. In order to mitigate the risk of extinction for the species, a Biodiversity Management Plan for Species (BMP-S) was compiled (Tarrant & Armstrong 2017) and gazetted by the Minister of Environmental Affairs of South Africa in June 2017. The BMP-S outlines a number of objectives aimed at the conservation of *H. pickersgilli* and identifies actions necessary to address the threats currently facing the species through implementation by various role players. These include rehabilitation of existing sites, and the facilitation of Biodiversity Stewardship or community stewardship agreements to help secure currently unprotected sites (Tarrant & Armstrong 2017). Additionally, conservation research, including long-term monitoring and development of *ex-situ* breeding programmes have been identified as part of these objectives (Tarrant & Armstrong 2017).

An additional objective of the BMP-S is to understand the possible effect of habitat fragmentation on the species' genetic structure in order to guide possible relocation or reintroduction efforts (Tarrant & Armstrong 2017). The aim of the current study, therefore, was to investigate the genetic diversity and population structure of *H. pickersgilli* using novel species-specific nuclear markers and mitochondrial DNA data in order to describe current, and infer historical, patterns of genetic structure. We hypothesise that, because of habitat fragmentation, barriers to gene flow resulting from physical separation and/or a lack of connectivity between various localities have resulted in groupings that may show some genetic distinction according to sampling locality. In addition, we predict that genetic diversity of *H. pickersgilli* may be low, because of habitat loss and fragmentation.

Materials and methods

Sampling and DNA isolation

A total of 48 *H. pickersgilli* toe clip samples were collected from 12 localities within the species' range (Supplementary Table 1, Fig. 2). Ethical approval for this project was obtained from the North-West University (NWU) and the Endangered Wildlife Trust (EWT). Permits for sampling were obtained from the provincial conservation authority, Ezemvelo KZN Wildlife (Permit No. OP 5080/2013). DNA was extracted using the DNeasy® Blood and Tissue Kit (QIAGEN) according to manufacturer extraction protocol.

Species-specific microsatellite development

Microsatellite enrichment was performed on three *H. pickersgilli* samples (AACRG2454, AACRG2461, AACRG2462) using Fast Isolation by AFLP of Sequences Containing repeats

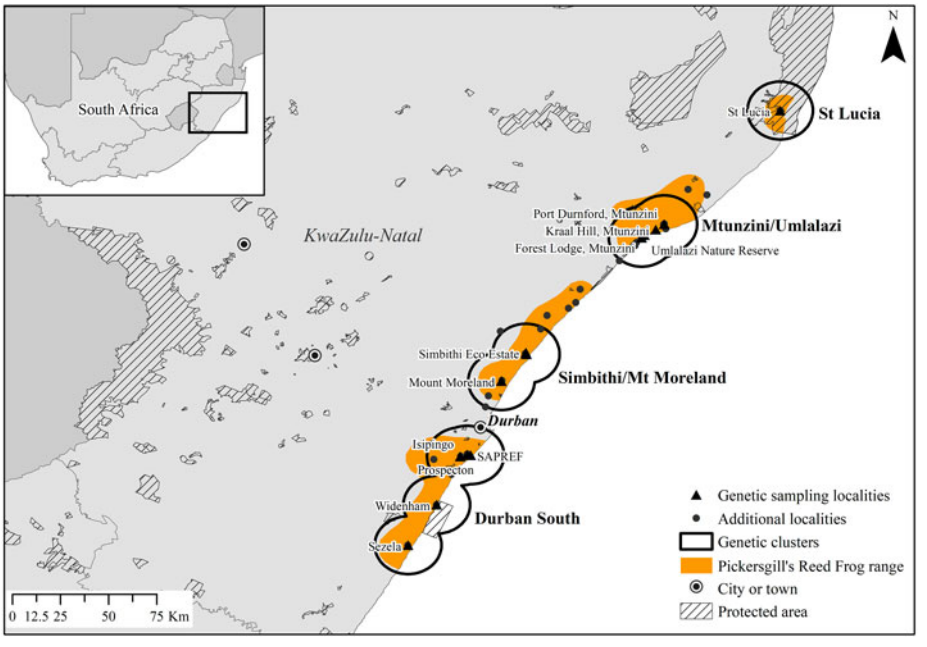

Figure 2. (A) Map of South Africa indicating the relative position of the KwaZulu-Natal province. (B) KwaZulu-Natal province showing the localities of *Hyperolius pickersgilli* samples used in the current study (triangles) in relation to precipitation (mm rainfall per year). Samples used in the current study originated (N→S) from: St Lucia; Port Durnford, Kraal Hill, Forest Lodge and Umlalazi Nature Reserve (Mtunzini cluster); Simbithi Eco Estate; Mount Moreland; and Prospecton, SAPREF, Isipingo, Widenham and Sezela (Durban South cluster).

(FIASCO) (Zane et al. 2002; Cortinas et al. 2006) with the following probes; $(AGGG)_4$, $(GTG)_5$, $(GTA)_5$, $(AC)_5$, $(AAAT)_5$, $(ATA)_5$, $(CT)_5$ and $(TGC)_5$. The microsatellite-enriched libraries (insert size 300–1 500 base pairs) were pooled and sequenced on the Illumina MiSeq PE300 platform at Inqaba biotec (Pretoria, Gauteng, South Africa). Sample preparation and analytical processing were performed at Inqaba biotec using their established protocols. The program MSATCOMMANDER version 0.8.1 (Faircloth 2008) was used to search the resulting reads for microsatellite motifs between two and six bp and with ≥8 repeats in length. A total of 53 microsatellite repeats were identified. Primers flanking repeat regions were designed using PRIMER 3 software for 26 loci (Rozen & Skaletsky 1997), and of these, 15 primer pairs were identified as polymorphic.

Mitochondrial DNA sequencing and microsatellite genotyping

Both mitochondrial and microsatellite markers were used in the current study. PCR amplification and sequencing were conducted for samples ($n = 34$) representing all 12 localities. Mitochondrial 16S was amplified using the following primer pairs: 16SA (5′-CGCCTGTTTATCAAAAACAT-3′) and 16SB (5′-CCGGTCTGAACTCAGATCACGT-3′) and the COI region was amplified and sequenced according to the protocols outlined by Hsieh et al. (2001). Sequences were edited and a concatenated 16S/COI dataset (1 212 nts) created using BioEdit v. 7.2.5 (Hall 1999). The best fit model of sequence evolution was selected using

jModeltest under the Akaike Information Criterion (AIC) (Posada 2008). Maximum likelihood and neighbour-joining analyses were carried out in MEGA6 (Tamura et al. 2013). Maximum likelihood was run under the GTR + I+G model with 1 000 non-parametric bootstrap replications using a heuristic search. Neighbour-joining analysis was run using the p-distance method with 1 000 non-parametric bootstrap replications using a pairwise deletion method. Bayesian inference was implemented in MrBayes v.3.1.2 (Huelsenbeck & Ronquist 2001) using four Markov chains for five million generations each, sampling every 100 generations, ensuring that the standard deviation of the split frequencies was less than 0.01. The chains were heated with the temperature scaling factor T = 0.02. The first 50 000 trees were discarded as burn-in, in each case having checked in a preliminary run that this was more than sufficient to achieve stationarity. A 50% majority-rule consensus tree was constructed from the remaining trees. *Hyperolius marmoratus*, *Heterixalus madagascariensis* and *Tachycnemis seychellensis* all belong to the Hyperoliinae subfamily and were selected as outgroups for analysis (Vences et al. 2003). Phylogenetic relationships between haplotypes were estimated using TCS v. 1.21 (Clement et al. 2000) to create a statistical parsimony network at 95% confidence. Haplotype and nucleotide diversity, neutrality tests, i.e. Fu & Li's F* and D* (Fu & Li 1993), Fu's Fs (Fu 1997) and Tajima's D (Tajima 1989), and population size change tests, i.e. mismatch distribution, raggedness (rg) and Ramos-Onsins and Rozas (R2) statistics, were performed on the full mitochondrial dataset (34 individuals combined) in DnaSP v. 5.10 (Librado & Rozas 2009) to search for signs of population expansion.

The 15 microsatellites for *H. pickersgilli* identified above were found to be suitably polymorphic for the purposes of the current study (as shown in Table 1). Amplifications were carried out in 15 µl reaction volumes containing 1X PCR buffer (Promega Corporation), 1.5–2.5 mM $MgCl_2$ (Promega Corporation), 200 µM deoxynucleoside-triphosphate mixture (dNTPs), 10 pmol of each primer (forward and reverse), 1 U μl^{-1} Promega GoTaq® Flexi DNA polymerase (Promega Corporation) and 10–20 ng genomic DNA template. The thermal cycling parameters were as follows: 95 °C for 5 min; 30 cycles of (95 °C for 30 s, 50–65 °C for 30 s and 72 °C for 30 s); 72 °C for 20 min. PCR products were pooled together and run against internal size standard Genescan™ 500 LIZ™ (Applied Biosystems Inc.) on an ABI 3130 Genetic Analyzer (Applied Biosystems). Samples were genotyped using GeneMapper v. 4.0. MICRO-CHECKER (Van Oosterhout et al. 2004) was used to detect possible genotyping errors, allele dropout and null alleles. Preliminary tests of pairwise F_{ST} distances showed no statistical significance between the 12 localities (Supplementary Table s 2 and 3); therefore localities separated by <30 km in distance were placed into four sub-population groups for analysis: (1) St Lucia ($n = 4$), (2) Mtunzini/Umlalazi cluster (Port Durnford, Kraal Hill, Forest Lodge and Umlalazi Nature Reserve) ($n = 9$), (3) Simbithi/Mt Moreland ($n = 19$) and (4) Durban South cluster (Prospecton, SAPREF, Isipingo, Widenham and Sezela) ($n = 16$) (Fig. 2). MS Toolkit (Park 2001) calculated the mean number of alleles per locus (A), observed heterozygosities (H_O), expected heterozygosities (H_E) and deviations from Hardy–Weinberg equilibrium (HWE), whereas GenAlEx (Peakall & Smouse 2012) was used to estimate the level of genetic diversity. Linkage disequilibrium between pairs of microsatellite loci within each species and locus was evaluated using GenePop v. 4.0 (Raymond and Rousset 1995). Associated probability values were corrected for multiple comparisons using Bonferroni adjustment for a significance level of 0.05. F_{ST}-based hierarchical analysis of molecular variance (AMOVA) was calculated using Arlequin

Table 1. List of species-specific microsatellite markers developed for *Hyperolius pickersgilli*.

Marker name	Forward	Reverse	Motif	Dye	Expected product Size (Bp)	Annealing temperature
PFG1	CTGGCAGGCAGATAGAGGC	ACAGCATCTTTGTGTGCAGC	AGAT	Ned	328	58 °C
PFG2	CTGAGTAACCCTTCCCACCC	CTTTGGGATGTGGTGGAACG	AGAT	Ned	186	50 °C
PFG4	GCCACATCGACCAGTGTTTG	CGTGTGTGCGGGCTATTTAC	AGAT	Vic	186	50 °C
PFG5	AAGTGATTTCAGGGAAGGGC	CAGGCAATGTCTCTGCTCAC	AGAT	Vic	307	58 °C
PFG7	TAACACAATGGACCTGCAGC	CCTAACAATGCACCGGGATG	AGAT	Fam	163	50 °C
PFG8	GCATAGGAAGGGACAGAGGG	AACGCTGCAAACCCACAAC	AGAT	Fam	299	58 °C
PFG9	CGGTATGGCCACCTTACAAC	TGGGTCTGAATCAGGAAAGG	AGAT	Pet	211	50 °C
PFG10	CCTGAGCCAAAATTATTGCAG	CTCCACACCAGGTCCGAATA	AGAT	Pet	170	50 °C
PFG13	GGCGCATCCTTATAAATGGC	GCTCCTGAGTTCTGTGATTGG	AGAT	Vic	167	50 °C
PFG14	TATGAAACGGGAGCCAGGAG	GTGTTCCAGTTCGCAACCTG	AGAT	Vic	217	50 °C
PFG16	TTGCATACATAATTGCAGCGAG	CTACAGACAAAGGAGCAGAGG	AGAT	Fam	224	50 °C
PFG17	CTCTGTCACTAGATCCCGACC	TAGACTTCAAGGGACCTGGC	AGAT	Fam	152	50 °C
PFG26	TGCTGGATCACTACTGTCGG	CCAGAACAAACAACACAAAGGG	AGAT	Ned	164	50 °C
PFG27	AAAGCGCTCCTAGTGGGTTT	TTCATGCGAGCAGAAAAATG	AGAT	Ned	232	50 °C
PFG33	TTGATTTACTGCGAGCCTGC	TATCCGGGACTTCTGTGTGG	AGAT	Pet	280	50 °C
PFG34	TTCAAACCTGCAAGCTCTCC	AACAGAGCGTGGAGAAGACC	ACTC	Pet	140	50 °C

v. 3.5.1.2 (Excoffier & Lischer 2010) to determine the level of genetic variation among and within the four sub-populations. STRUCTURE v. 2.3.3 (Pritchard et al. 2000) was used to infer the pattern of genetic structure among locality groups via Bayesian clustering analysis. Analysis was conducted where 4 runs for each *K* (1–12) were implemented with a Markov Chain Monte Carlo run-length of 500 000 generations, following a burn-in period of 20 000 iterations. Assessments were conducted without prior population information (option USEPOPINFO = 0). Posterior probabilities were calculated from the averaged four values for the estimated ln(Pr(X|K)). STRUCTURE HARVESTER (Earl & vonHoldt 2012) was used to determine the *K* with the greatest increase in posterior probability (Δ*K*), i.e. the most likely number of sub-populations, following the Evanno method (Evanno et al. 2005).

Results

Phylogenetic analysis

Maximum likelihood, neighbour-joining and Bayesian analysis of concatenated mitochondrial 16S and COI regions revealed congruence across their tree topologies. A lack of distinct phylogenetic structure was observed among the *H. pickersgilli* populations (Fig. 3). The *H. pickersgilli* samples were monophyletic with respect to the out-groups with strong support (100% bootstrap support; 1.00 posterior probability). The majority of branches within *H. pickersgilli* were poorly supported with most bootstrap values below 70%. Pairwise genetic distances between individuals had similar values, ranging between 0% and 1.2%, with the highest genetic distances observed between samples St Lucia-HP50 and SAPREF-HP45. A lack of divergence (0%) was observed between the four sub-population groups (localities separated by <30 km in distance). Analysis in TCS identified a total of 15 haplotypes using concatenated 16S and COI data (Fig. 4). An interconnected median-joining network depicts H2 (15 samples; 8 localities) and H11 (6 samples; 5 localities) as the most common haplotypes. Nucleotide diversity (π) values for both mitochondrial regions are particularly low (0.003), whereas haplotype diversity (*h*) is relatively higher (0.786). *Hyperolius pickersgilli* may be an expanding population, because models of demographic population expansion follow expected trends of high haplotype diversity and low nucleotide diversity (Slatkin & Hudson 1991). Additionally, the mismatch distribution analysis (Fig. 5) showed a unimodal pattern and both raggedness and R2 statistics were low (although not significant), indicating that the curve fits the sudden expansion model. Tajima's D statistic was significantly negative, indicative of recent population expansion; however Fu's Fs was not significant and Fu & Li's D* and F* were significantly negative, which does not meet the expectations for demographic expansion (Peck & Congdon 2004).

Genetic diversity and population structure

A total of 235 microsatellite alleles were observed from 48 samples, with the number of alleles per locus ranging from 11 to 23, with an average of 15.7 alleles per locus. Absence of null alleles was observed, linkage disequilibrium was not detected and groupings did not show any significant deviation from HWE (Table 2). The mean H_O value for

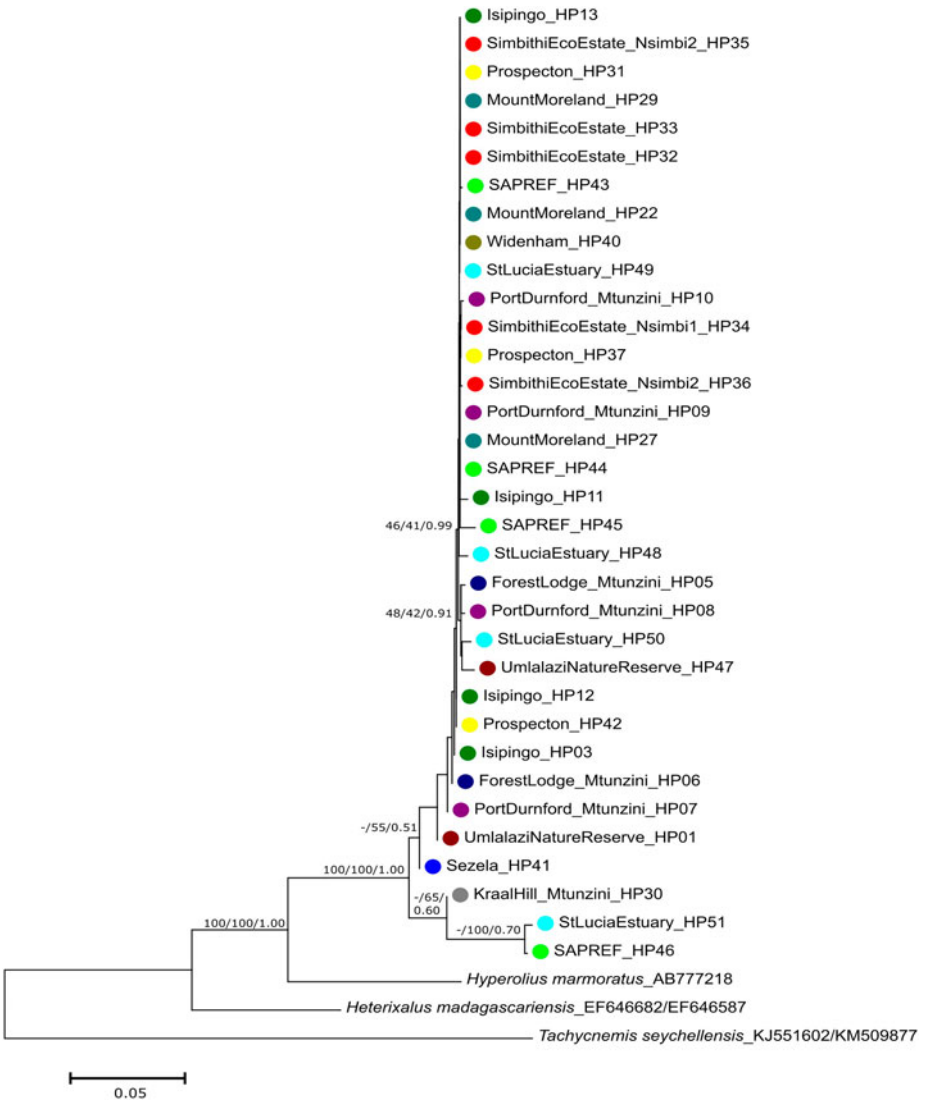

Figure 3. Phylogenetic tree based on 1 212 nucleotides of 16S and COI concatenated sequence data depicting the relationship between 34 samples of *Hyperolius pickersgilli* with respect to the various outgroups *H. marmoratus*, *Heterixalus madagascariensis* and *Tachycnemis seychellensis*. Analysis on the full mitochondrial dataset was conducted per individual. Bootstrap and posterior probability support is given at the nodes.

populations across all loci was 0.773 with the highest value found in the Simbithi/Mt Moreland (0.809). Mean H_E and uH_E values were 0.808 and 0.865, respectively, with the highest values observed in the Durban South cluster (H_E: 0.864; uH_E: 0.896). Expected heterozygosity values (H_E) were lowest in St Lucia (0.682), which comprises fewer samples, and the highest in groups with greater sample numbers, i.e. Simbithi/Mt Moreland (19 samples) and Durban South (16 samples). The highest allelic richness (AR) (3.427) and F_{IS} (0.140) values were found in the Durban South cluster. F_{IS} values were not significant,

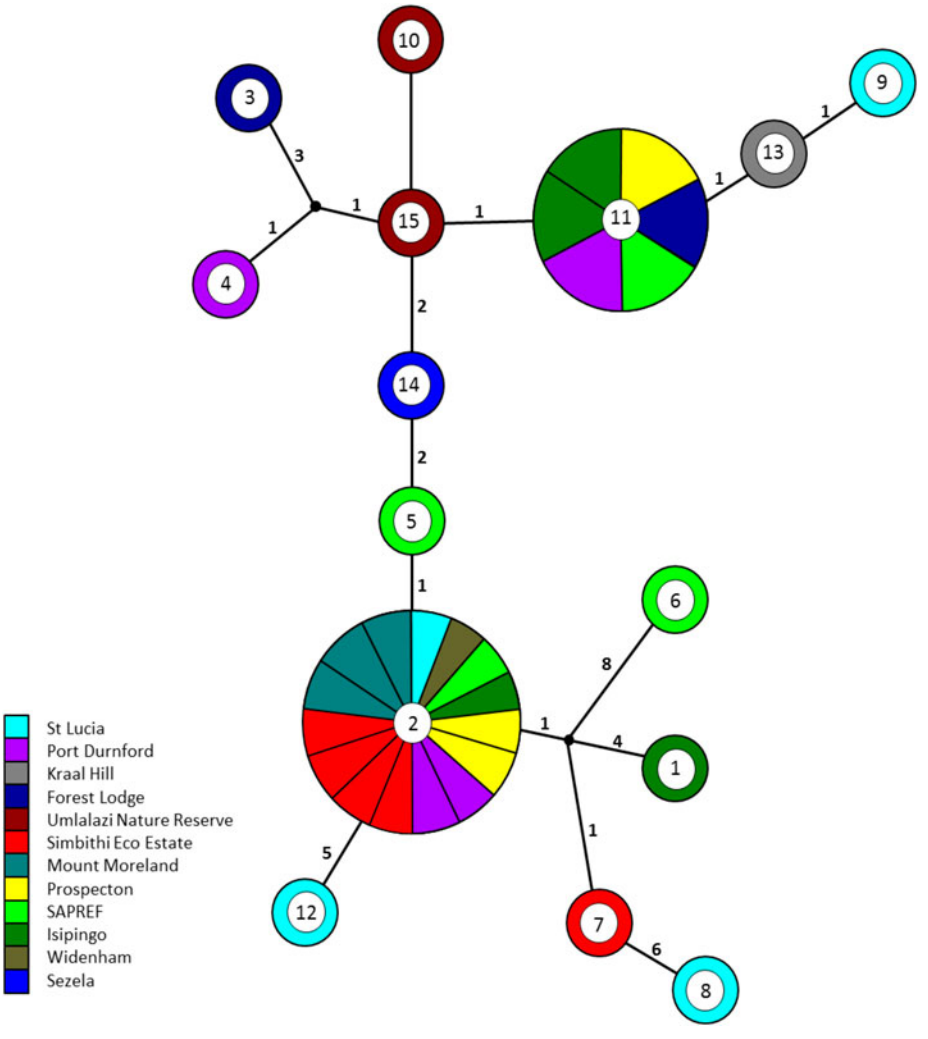

Figure 4. Median-joining, statistical parsimony haplotype network for 16S and COI concatenated data. Circles represent the various haplotypes (numbered) and colours indicate sampling localities. Connecting dots represent missing haplotypes.

which may indicate a lack of inbreeding within these sub-populations. Pairwise F_{ST} values ranged from 0.030 (Simbithi/Mt Moreland–Durban South) to 0.091 (Simbithi/Mt Moreland–St Lucia) with the highest average distance found between St Lucia and the other populations (0.087). Average pairwise F_{ST} showed that Durban South had the lowest distance from other populations (0.053). Analysis of molecular variance (AMOVA) revealed the greatest source of variation in *H. pickersgilli* to be within individuals (75%), and the lowest source among populations (3%). 22% of variance is accounted for by variation among individuals. F_{IS} (0.222), F_{ST} (0.030) and F_{IT} (0.246) statistics for the full data set were each significant (p (random value $\geq$ observed value) = 0.001).

The genetic relationship between the sub-populations, inferred via STRUCTURE, identified $K = 2$ as the most probable K value (Fig. 6), where $K = 2$ (Supplementary Fig. 4)

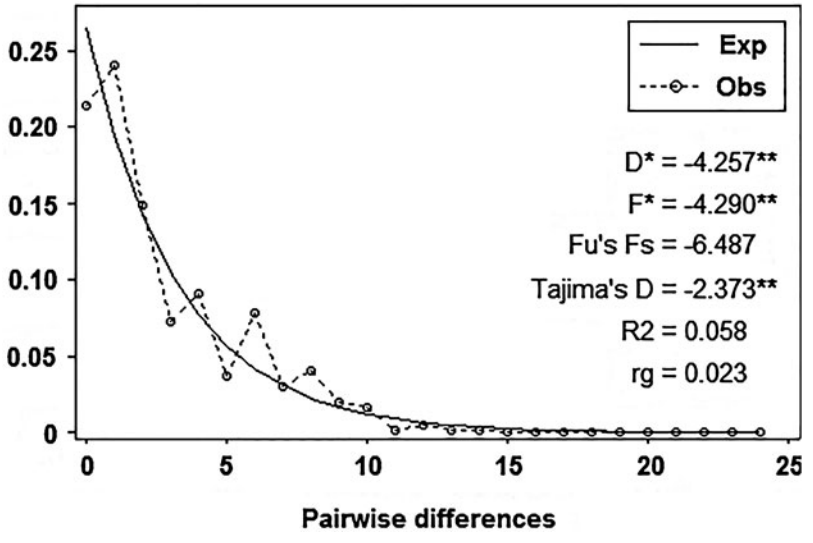

Figure 5. Observed and expected mismatch distribution under the sudden population expansion model for *Hyperolius pickersgilli* throughout the KwaZulu-Natal region. Neutrality test results for Fu & Li's D* ($p < 0.02$) and F* ($p < 0.02$), Fu's Fs, and Tajima's D ($p < 0.01$) are included, as well as raggedness (rg) and Ramos-Onsins and Rozas (R2) based on mitochondrial 16S and COI concatenated data.

Table 2. Microsatellite-based pairwise F_{ST} values estimated during AMOVA (above diagonal) and pairwise distances based on concatenated mtDNA 16S and COI data (below diagonal) for four locality groupings: (1) St Lucia, (2) Mtunzini/Umlalazi (Port Durnford, Kraal Hill, Forest Lodge and Umlalazi), (3) Simbithi/Mt Moreland and (4) Durban South (Prospecton, SAPREF, Isipingo, Widenham and Sezela). Outgroups *Hyperolius marmoratus*, *Heterixalus madagascariensis* and *Tachycnemis seychellensis* are included for mtDNA pairwise genetic distances.

		1	2	3	4	5	6	7
1	St Lucia	–	0.086	0.091	0.085	n/a	n/a	n/a
2	Mtunzini/Umlalazi	0.000	–	0.048	0.044	n/a	n/a	n/a
3	Simbithi/Mt Moreland	0.000	0.000	–	0.030	n/a	n/a	n/a
4	Durban South	0.000	0.000	0.000	–	n/a	n/a	n/a
5	*H. marmoratus*	0.122	0.123	0.123	0.123	–	n/a	n/a
6	*H. madagascariensis*	0.145	0.145	0.146	0.145	0.141	–	n/a
7	*T. seychellensis*	0.399	0.399	0.400	0.399	0.386	0.346	–

displayed the greatest posterior probability (Evanno et al. 2005). However, given the lack of genetic structure observed, K = 1 may represent the most probable number of groups, but because of the nature of the analysis, ΔK cannot be $K = 1$ (Earl & vonHoldt 2012).

Discussion

Genetic structure

The current study sought to examine the genetic structure of the range-restricted species *H. pickersgilli* using both mitochondrial and nuclear DNA data. *Hyperolius pickersgilli* populations inhabit a very small total area, with an AOO of only 12 km^2 spread across the coast line of KwaZulu-Natal province of South Africa (IUCN 2016). Complex spatial structure over

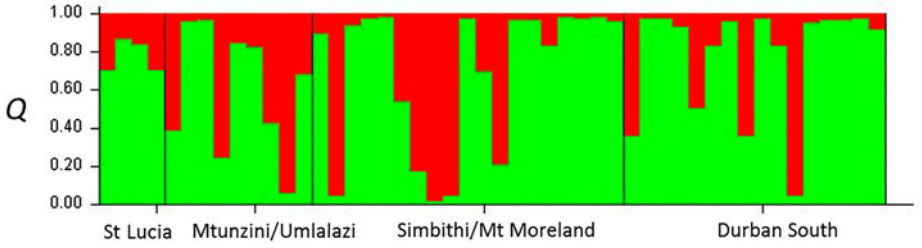

Figure 6. Graph illustrating probabilities of assignment of individuals to different genetic clusters (*Q*) ($K = 2$) based on microsatellite genotypes. Each individual is represented by a single vertical line and each colour within the column represents the relative likelihoods of that individual belonging to each of the two defined clusters. Numbers on the *x*-axis correspond to the various samples and their respective localities: (1) St Lucia, (2) Mtunzini/Umlalazi (Port Durnford, Kraal Hill, Forest Lodge and Umlalazi), (3) Simbithi/Mt Moreland and (4) Durban South (Prospecton, SAPREF, Isipingo, Widenham and Sezela).

shorter distances has been reported in other amphibian species, because of their poor dispersal ability and the presence of physical barriers, such as elevational difference, ridges and/or watersheds (Funk et al. 2005; Angelone et al. 2011). Contrary to the expectation of genetic separation according to the geographical locality, genetic analysis shows that there is no specific phylogeographic structure among *H. pickersgilli* individuals sampled across the 12 localities. Maximum likelihood, neighbour-joining and Bayesian analysis show the clear separation of *H. pickersgilli* from the various outgroups, as a single clade with relatively poorly supported subgroups (Fig. 3). Internal structuring according to geographical location is absent and samples appear to be randomly associated (Fig. 3). These findings are also supported by the median-joining haplotype network that displays sharing of mitochondrial haplotypes among localities (Fig. 4). The haplotype network consists of 15 haplotypes from 34 samples with the two major shared haplotypes comprising individuals from various localities across the range, e.g. haplotype 2 comprised samples from iSipingo wetland, Mount Moreland, Port Durnford, Durban South, SAPREF, Simbithi Eco-Estate, St Lucia and Widenham (Fig. 4). Low genetic diversity based on mtDNA analysis, coupled with the high frequency of a single haplotype, may be indicative of a recent radiation of the species (Hewitt 1996, 2000), which would suggest a more recent occupation of the range. Although expansion may be a possibility, as evidenced by the unimodal mismatch distribution (Fig. 5), significantly negative Tajima's D and low nucleotide diversity coupled with high haplotype diversity, does not support this assertion. Hence the observation of the presence of a high frequency haplotype could potentially have arisen, because of a different process, such as a selective sweep. Results suggest the existence of recent past or present gene flow between individuals from the various localities. Although the distribution of *H. pickersgilli* has become more fragmented with recent landscape changes that have altered the species' habitat (Measey 2011), these changes have occurred over a relatively short timeframe of a few decades and do not appear to be sufficient to produce observable genetic differences.

Microsatellite genotype analysis is consistent with the mitochondrial data, indicating limited genetic structure ($K = 2$). The STRUCTURE bar plot shows admixture of genotypes and illustrates the random distribution of assignment probabilities across the 12 populations (Fig. 6). This suggests that there is gene flow between the various populations,

resulting in the lack of genetic structure observed. This finding is supported by low pairwise F_{ST} values between the defined sub-populations (ranging from 0.030 to 0.091) (Table 3). Although several private alleles were observed in samples from each locality, this is more likely to be because of the high number of alleles per marker in combination with low sample numbers. Additionally, an analysis of molecular variance (AMOVA) revealed the greatest source of variation to be within individuals (75%), whereas variation among populations accounts for the lowest percentage (3%) (Table 4). The lack of significant deviation from HWE also points to the maintenance of gene flow among populations (Table 3).

Both mitochondrial and nuclear data accordingly refute the hypothesis that *H. pickersgilli* samples separate according to geographical locality on a local scale, because of habitat fragmentation. Weak genetic structure is a common trend found in amphibian populations appearing on small geographic scales with limited distributions (Rowe et al. 2000). In addition, the lack of topographic complexity in this coastally restricted species is consistent with the lower genetic divergences observed within many lowland frog species (Guarnizo & Cannatella 2013; Rodríguez et al. 2015). Results from the current study indicate that gene flow between *H. pickersgilli* localities is not restricted or has at least occurred in the relatively recent past. Current knowledge on the distribution of *H. pickersgilli*, authenticated by surveys guided by predictive modelling (Tarrant & Armstrong 2013), documents the addition of new subpopulations, as well as a minimal increase in area of occupancy (from 9 km^2 to 12 km^2), accordingly supporting the hypothesis that the range was fairly continuous in the recent past.

Genetic diversity

The ability to adapt to ever-changing environmental conditions is afforded by the genetic diversity of an organism, and populations lacking genetic diversity may be unable to adapt to environmental change (Frankham et al. 2002). *Hyperolius pickersgilli* groups display similar diversity values with a relatively high average H_O of 0.773 across all samples (Table 3). These values are similar to demographically sTable species ($H_O \approx 0.85$; Allendorf & Luikart 2007). Moderate to high observed heterozygosity coupled with low, non-significant inbreeding coefficients (F_{IS}) (Table 3) suggest a relatively high level of genetic diversity within *H. pickersgilli*. *Hyperolius pickersgilli* has therefore been able to maintain relatively high levels of genetic diversity, despite landscape changes. Although the

Table 3. Descriptive statistics averaged across all loci for *Hyperolius pickersgilli* from four locality groupings: (1) St Lucia, (2) Mtunzini cluster (Port Durnford, Kraal Hill, Forest Lodge and Umlalazi), (3) Simbithi/Mt Moreland and (4) Durban South cluster (Prospecton, SAPREF, Isipingo, Widenham and Sezela). Number of samples (*n*), number of alleles (*N*), number of effective alleles (N_E), observed heterozygosity (H_O), expected heterozygosity (H_E), unbiased expected heterozygosity (uH_E), allelic richness (AR), inbreeding coefficient (F_{IS}) and deviation from Hardy–Weinberg equilibrium (HWE). NS = non-significant deviation.

Population	*n*	*N*	N_E	H_O	H_E	uH_E	AR	F_{IS}	HWE
St Lucia	4	4.200	3.455	0.717	0.682	0.796	2.979	0.119	NS
Mtunzini/Umlalazi	9	7.867	5.736	0.788	0.821	0.878	3.331	0.108	NS
Simbithi/Mt Moreland	19	16.067	7.758	0.809	0.863	0.891	3.414	0.094	NS
Durban South	16	14.400	7.725	0.777	0.864	0.896	3.427	0.140	NS
Mean		10.634	6.169	0.773	0.808	0.865	3.288	0.115	NS

Table 4. Analysis of Molecular Variance among and within *Hyperolius pickersgilli* individuals from 12 South African populations. Fixation indices were as follows: $F_{IS} = 0.222$, $F_{ST} = 0.030$, $F_{IT} = 0.246$. Based on 16 000 permutations, all values were significant ($p < 0.01$).

Source of variation	DF	Sum of squares	Variance components	Percentage variation
Among populations	3	38.086	0.207	3
Among individuals	44	357.070	1.474	22
Within individuals	48	248.000	5.167	75
Total	95	643.156	6.848	100

KwaZulu-Natal coastal area has been surveyed for various amphibian fauna, the detection of *H. pickersgilli* in the wild can be difficult, because of its inconspicuous nature (Raw, 1982; Tarrant & Armstrong 2013). This cryptic nature, coupled with the recent discoveries of *H. pickersgilli* in new localities (thereby increasing the area of occurrence) indicates that there are likely additional localities that have not yet been discovered. The protection and management of these habitats is necessary to provide corridors for these populations and accordingly maintain high levels of gene flow. Although *H. pickersgilli* currently displays high genetic diversity, it is likely that landscapes will continue to change with continued urbanisation, mining, and other land uses. Although the cultivation of monocultures on wetlands is prohibited in South Africa (Conservation of Agricultural Resources Act No. 43 of 1983), the ongoing loss of habitat along the KwaZulu-Natal coast is pervasive and results not only in direct loss of breeding sites, but in the loss of habitat corridors necessary to promote movement between the sites. *Hyperolius pickersgilli*, because of its restricted habitat, is sensitive to environmental change (Russell & Downs 2012).

The case of *H. pickersgilli* clearly demonstrates the challenges involved in amphibian conservation. One of the key requirements for species management is the need to identify and gather information regarding the abundance and distribution of individuals and/or populations of the species across their range (Shaffer et al. 2015). Although data collection is possible, the cryptic nature of most amphibians requires intensive sampling and survey effort over several years/generations (Beebee and Griffiths 2005; Shaffer et al. 2015). Additionally, constant resampling and resurveying needs to be done to provide better estimates of temporal changes in population numbers and distribution (Skelly et al. 2003). Identifying key populations can aid in identifying potential areas for protection (Petit et al. 1998), especially in the case of widespread species, however this can be problematic for those species with limited range.

Species with limited distributions have a relatively smaller range but not all areas within the range can be designated as areas needing protection. Those groups outside of reserves will be subject to a higher level of threat, especially where habitat destruction is concerned, because small losses in habitat have greater impact for those species with restricted ranges (Pimm et al. 2014). Additionally, some frog species, e.g. *Rana catesbeiana* (Austin et al. 2004), *R. temporaria* (Brede & Beebee 2004) and *H. pickersgilli* from this investigation, show evidence of gene flow. This raises the question of how best to define key populations, ESUs (evolutionary significant units) and/or MUs (management units) (Shaffer et al. 2015) as priorities for protection, especially when there is a clear lack of genetic distinction or groupings among individuals.

Combining genetic analyses with field-based demographic studies may be the most effective approach for the development of conservation management plans (Shaffer et al. 2015). Increased understanding of populations and their structure, dynamics and behaviours, including migration, expansion or decline, may help identify priority populations and/or areas for species with limited distributions (Marsh & Trenham 2001). With this in mind, it is apparent that there is a need for continued studies, both genetic and ecological, on the *H. pickersgilli* population of South Africa. Long-term studies/observations, as well as monitoring of species response to additional habitat change and/or conservation efforts, will help officials implement more effective management plans, including the identification and preservation of priority habitats. Conservation of this endangered species should remain a top priority in order to maintain current, representative levels of genetic diversity.

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