

Phylogeography of the Clicking Stream Frog *Strongylopus grayii* (Anura, Pyxicephalidae) reveals cryptic divergence across climatic zones in an abundant and widespread taxon

Krystal A. Tolley, Anne Braae & Michael Cunningham

To cite this article: Krystal A. Tolley, Anne Braae & Michael Cunningham (2010) Phylogeography of the Clicking Stream Frog *Strongylopus grayii* (Anura, Pyxicephalidae) reveals cryptic divergence across climatic zones in an abundant and widespread taxon, African Journal of Herpetology, 59:1, 17-32, DOI: [10.1080/04416651003744943](https://doi.org/10.1080/04416651003744943)

To link to this article: <https://doi.org/10.1080/04416651003744943>



Published online: 15 Jun 2010.



Submit your article to this journal [↗](#)



Article views: 363



View related articles [↗](#)



Citing articles: 2 View citing articles [↗](#)

Original article

Phylogeography of the Clicking Stream Frog *Strongylopus grayii* (Anura, Pyxicephalidae) reveals cryptic divergence across climatic zones in an abundant and widespread taxon

KRYSTAL A. TOLLEY^{1*}, ANNE BRAAE^{1,3} &
MICHAEL CUNNINGHAM²

¹*Applied Biodiversity Research Division, South African National Biodiversity Institute, Cape Town, South Africa;* ²*Department of Zoology, University of the Free State, Phuthaditjhaba, South Africa;* ³*Department of Zoology, University of Cape Town, Cape Town, South Africa*

Abstract.—The Clicking Stream Frog (*Strongylopus grayii*) occurs throughout southern and eastern South Africa across summer, aseasonal and winter rainfall areas. Because of its contiguous distribution and extended breeding season this species might be expected to show a phylogeographic pattern of isolation by distance rather than divergent geographical lineages. To investigate population structure and make inferences about physical processes that have influenced gene flow in this species, two mitochondrial genes (ND2 and 16S) were sequenced for 64 frogs from across the distribution of the species. Phylogenetic analyses (parsimony and Bayesian) revealed two well-supported clades partitioning the range of this taxon, with one clade restricted to the northeast (summer rainfall) and the other restricted to the south (winter and aseasonal rainfall). The magnitude of sequence divergence between these clades is higher than expected, and is typical of levels of sequence divergence found among closely related species of pyxicephalid frogs. Phylogeographic analyses of sequence networks and spatial analysis of molecular variance (SAMOVA) revealed additional geographical structure within each of these lineages. This study demonstrates the effectiveness of climatic transitions as barriers to gene flow and as potential agents for vicariant speciation.

Key words.—South Africa, amphibian, phylogeny, vicariance, speciation, climatic barriers

INTRODUCTION

New amphibian species are still being discovered, with species numbers increasing world-wide by over 25% in the past 11 years (Köhler *et al.* 2005). This increase is largely due to intensified tropical exploration and the use of molecular techniques to identify previously unknown lineages (Köhler *et al.* 2005; Bickford *et al.* 2007). Often, cryptic species are recognised only through the use of molecular techniques to identify genetically distinct lineages (Stuart *et al.* 2006; Fouquet *et al.* 2007; Vieites *et al.* 2009). Additionally, incorporating information on the geographical distribution of genetic lineages within taxa allows predictions to be made about the historical biogeography and organismal evolution of species and species groups (Avice 2000;

*Corresponding author. Email: k.tolley@sanbi.org.za

Beheregaray 2008; Buckley 2009). Perhaps more importantly, these patterns provide clues as to the historical processes that have created hotspots of endemism. Ongoing improvements in our understanding of diversity patterns will allow better land-use planning which can provide better protection of biological diversity, despite ongoing loss of habitat (Beheregaray 2008).

A vital component of phylogeographic studies is the identification of historically independent lineages where morphological differences are not obvious despite substantial genetic divergence (Beheregaray & Caccone 2007). In many cases, these cryptic lineages qualify as distinct species, some of which do show consistent differences that have been confused with variability within species (Dawood *et al.* 2002; Measey *et al.* 2007; Channing & Schmitz 2008; Blackburn 2009). This is particularly important where cryptic taxa co-occur or where sister species meet on either side of a biogeographic barrier that potentially affects multiple taxa. Essentially, phylogeographic studies of wide-ranging, geographically fragmented, phenotypically or ecologically variable taxa can be used to ensure that current methods for evaluating biodiverse areas, such as tallies of species diversity and endemism, take into account the diversity that is not accessible through existing morphologically-oriented taxonomic assessments. There is a deficiency of such phylogeographic studies in megadiverse countries, including South Africa, and especially in biodiversity hotspots (Beheregaray 2008), such as the Cape Floristic Region and the Maputaland–Pondoland–Albany complex. This study contributes to addressing these needs through a phylogeographic analysis of a wide-ranging species that spans these global biodiversity hotspots.

Study Species

Relationships within ranoid frogs inferred on the basis of genetic evidence suggest that a combination of conserved and homoplasious morphological characters, along with uneven morphological change among genera, may have confounded traditional taxonomy (Van der Meijden *et al.* 2005; Bossuyt *et al.* 2006; Frost *et al.* 2006). Based on nuclear and mitochondrial DNA sequence data, Frost *et al.* (2006) transferred all southern African frogs previously included in the family Ranidae, including *Strongylopus*, to the newly recognised family Pyxicephalidae. Although there is general consensus that these frogs form an African endemic clade, the assignment of this group to familial or sub-familial taxonomic levels remains controversial, due to the associated taxonomic changes in other ranoid frogs (Hillis 2007; Frost *et al.* 2008).

The genus *Strongylopus* has also undergone taxonomic reassessment, due to the recognition of geographically separated forms as full species and the reassignment of one species to another genus (Channing & Davenport 2002; Poynton 2004; Clarke & Poynton 2005; Tarrant *et al.* 2008). Currently ten species are recognised from this genus (Frost 2009). Several species in *Strongylopus* are cryptic or difficult to identify on morphology alone (Minter *et al.* 2004). For example, *S. springbokensis* Channing, 1986 from Namaqualand in northwestern South Africa is morphologically very similar to *S. grayii*, differing only in its broader snout, but is easily distinguished by its mating advertisement call (Channing 1986; see also summary in du Preez &

Carruthers 2009). *Strongylopus rhodesianus* (Hewitt, 1933) from the Chimanimani highlands of eastern Zimbabwe is also very similar to *Strongylopus grayii*, differing only in its more elongate snout, in the extent of nuptial pads and in calls, with both of the latter characters being secondary sexual characteristics of mature males (Hewitt 1933; du Preez & Carruthers 2009). Several studies have suggested the possibility of additional geographically separate cryptic taxa within *Strongylopus grayii* (Jacobsen 1989; Pickersgill 2007) although these were related to geographic locations and habitat rather than climatic zones.

Strongylopus grayii is widely distributed throughout southern and eastern South Africa, from the Western Cape Province eastward through the Eastern Cape Province and Lesotho to KwaZulu-Natal and the eastern Free State, and northwards along the inland escarpment, through Swaziland and Mpumalanga to Limpopo Province (Fig. 1; Channing 2004). This encompasses an unusually broad altitudinal range, from sea level to 3 200 m at latitude 29°S, but restricted to lower altitudes in the southwest and excluded from lower altitudes in the northeast. This distribution also traverses three distinct rainfall regimes: winter (southwest), aseasonal (south) and summer (east). Calling occurs throughout the year around any available still water, although most breeding occurs in the wettest months in each of these rainfall zones (Channing 2004). Thus, a temporal discontinuity in breeding could potentially restrict gene flow in this species. Although *S. grayii* is widely distributed, abundant

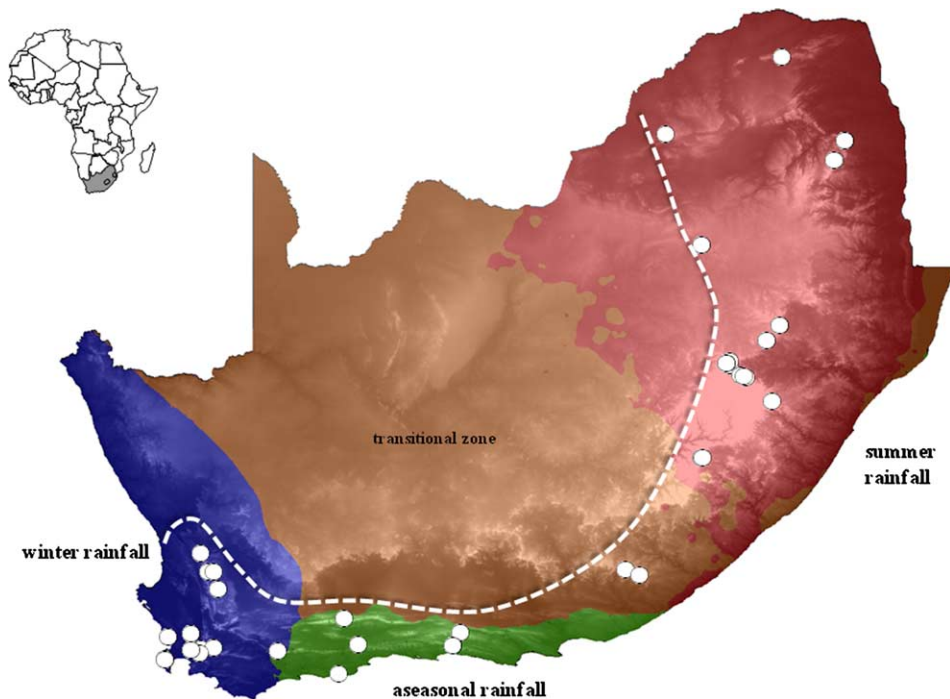


Figure 1. Distribution of *Strongylopus grayii* superimposed over a digital elevation map and rainfall zones, with sampling localities shown as dots. The white dotted line demarcates the approximate distribution (distribution data source Minter et al. 2004).

and easily sampled, there have been no previous studies of molecular genetic variation in this species.

In this study, maternally inherited mitochondrial (mt) DNA sequences are used to infer historical patterns of female gene flow in this species. We used these data to evaluate support for several competing hypotheses, either that (1) *S. grayii* is a single widely distributed species without appreciable genetic structuring, (2) that genetic divergences among *S. grayii* populations correlate with distances among sampling localities rather than with environmental differences, and (3) *S. grayii* comprises distinct lineages in different climate zones, which may be cryptic species. To evaluate these hypotheses we sequenced two regions of mtDNA, 16S rRNA and the NADH 2 (ND2) gene. We interpret our results in the context of this species, but also within a wider context that considers potentially congruent and/or discordant patterns in other species across these climate zones in order to enhance our understanding of the overall South African biota.

MATERIALS AND METHODS

Tissue samples (single phalange clips, or tadpole tails) were obtained during general herpetological field surveys between 2000 and 2008, and were stored in 99% ethanol. In total, 64 samples were obtained from throughout the distribution range of *S. grayii* although sampling gaps exist in the eastern and northern parts of the range. DNA was extracted using the Qiagen DNAEasy extraction kit (Qiagen, Valencia, USA) or by salt extraction.

Two mitochondrial fragments (ND2 and 16S) were amplified using general lab protocols for PCR and sequencing. The ND2 gene was amplified using the vMet2-vTrp primer set (Cunningham & Cherry 2004) and 16S was amplified using the 16Sa and 16Sb primer pair (Palumbi 1991). Each PCR reaction was set up in 50 µl reaction volumes, as follows: 1X thermophilic reaction buffer (Promega, Madison, USA), 1µM MgCl₂, 0.4 µl dNTPs (at 10 µM of each dNTP), 0.04 µM of each ND2 primer, or 0.2 µM of each 16S primer, and 2 units *Taq* polymerase (Promega, Madison, USA). Between 4 and 6 µl of genomic DNA was used as template depending on template concentration (usually between 10–30 ng/µl). PCR was performed using the following reaction profile: an initial denaturing of 94 °C for 1 min 30 sec, followed by 34 cycles of 94 °C for 45 sec, 55 °C for 45 sec (ND2) or 52 °C for 30 sec (16S), and 72 °C for 1 min 30 sec, with a final extension step of 72 °C for 5 min.

Amplified PCR products were run on a 0.8% agarose gel stained with ethidium bromide and visualised by UV trans-illumination. The products were either sent directly to Macrogen Corp. (Seoul, Korea) for clean up and sequencing, or were purified using the Qiagen PCR clean-up kit (Qiagen, Valencia, USA), before being sent for sequencing. Sequencing was carried out using either the vMet2 or 16Sa primers, depending on the fragment amplified. Sequences were screened and edited using Sequence Navigator v1.01 (Perkin Elmer, Applied Biosystems, Foster City, USA), with sequence alignment performed in ClustalX using default alignment parameters (Thompson *et al.* 1997). The aligned sequences for both markers were checked in MacClade v4.0 (Maddison & Maddison 1992) for misalignments. All sequences have been deposited in GenBank (Accession numbers EF136505-136563 and GU 951994-GU 952079).

Phylogenetic Analyses

The combined dataset consisted of 64 individuals and 1 379 characters (553 bp of 16S, 826 bp of ND2). A partition homogeneity test, implemented in PAUP v4.0b10 (Swofford 2002), did not indicate any conflicting phylogenetic signal between the two mitochondrial genes ($p > 0.05$, 100 repartitions), so the genes were combined into a single dataset (Hipp *et al.* 2004). Two methods of phylogenetic reconstruction were implemented on this combined dataset; maximum parsimony (MP) and Bayesian inference (BI). In each case, the outgroup consisted of two individuals of *Strongylopus fasciatus*.

For the parsimony search, a heuristic, unweighted search was implemented in PAUP 4.0b10 (Swofford 2002), using the tree-bisection-reconnection (TBR) branch swapping algorithm, 1 000 random addition replicates with 100 trees held each replicate. Nodal support was assessed by 1 000 bootstrap replicates, each with 100 random addition replicates and 50 trees held for each replicate.

Bayesian analysis was performed in MrBayes version 3.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Modeltest 3.06 (Posada & Crandall 1998) indicated that the best fitting model of sequence evolution was either TrN + I + G (hLRT) or HKY + I + G (AIC). Therefore, the analysis was run with four rate categories with uniform priors for all parameters. Four data partitions were created: 16S, and 1st, 2nd, 3rd codons for ND2. These partitions were unlinked and allowed to optimise separately. To ensure the results converged on the same topology, the MCMC search was run twice with parallel runs for 10 million generations. Each parallel search used four Markov chains (one cold, three heated) initiated from a random tree, with trees saved every 1 000 generations. The burn-in of 3 500 trees was determined by examining stationarity of log likelihood tree scores and standard deviation of split frequencies using simple plots. In addition, the effective sample size (> 200) for all parameters was checked using Tracer v1.4.1 (Rambaut & Drummond 2007). The remaining 6 500 trees were used to construct a 50% majority rule consensus trees in PAUP 4.0b10. Nodes were considered supported with ≥ 0.95 posterior probabilities.

To give a rough indication of the degree of divergence between clades, sequence divergences were estimated using uncorrected p-distances in MEGA 2.1 for 16S and ND2 (Kumar *et al.* 2001). Intra-clade diversity was estimated from haplotype (h) and nucleotide (π) diversity (Tajima 1983; Nei 1987) using Arlequin v2.0 (Schneider *et al.* 2000).

Population Level Genetic Analyses

For population level analyses the dataset was trimmed to 55 individuals that had complete sequences for both mtDNA fragments (1 173 bp combined). Phylogenetic analyses revealed geographically separated clades within *S. grayii*, but also revealed a number of shallow polytomies within each clade (see Results) suggesting population level analyses would be suitable. Networks, diagrams that allow multiple pathways of connectedness among nodes, provide better representations of uncertainties in the inference of infraspecific gene genealogies than do bifurcating trees (Posada & Crandall 2001; Holland *et al.* 2004). Thus, to examine the relationships among

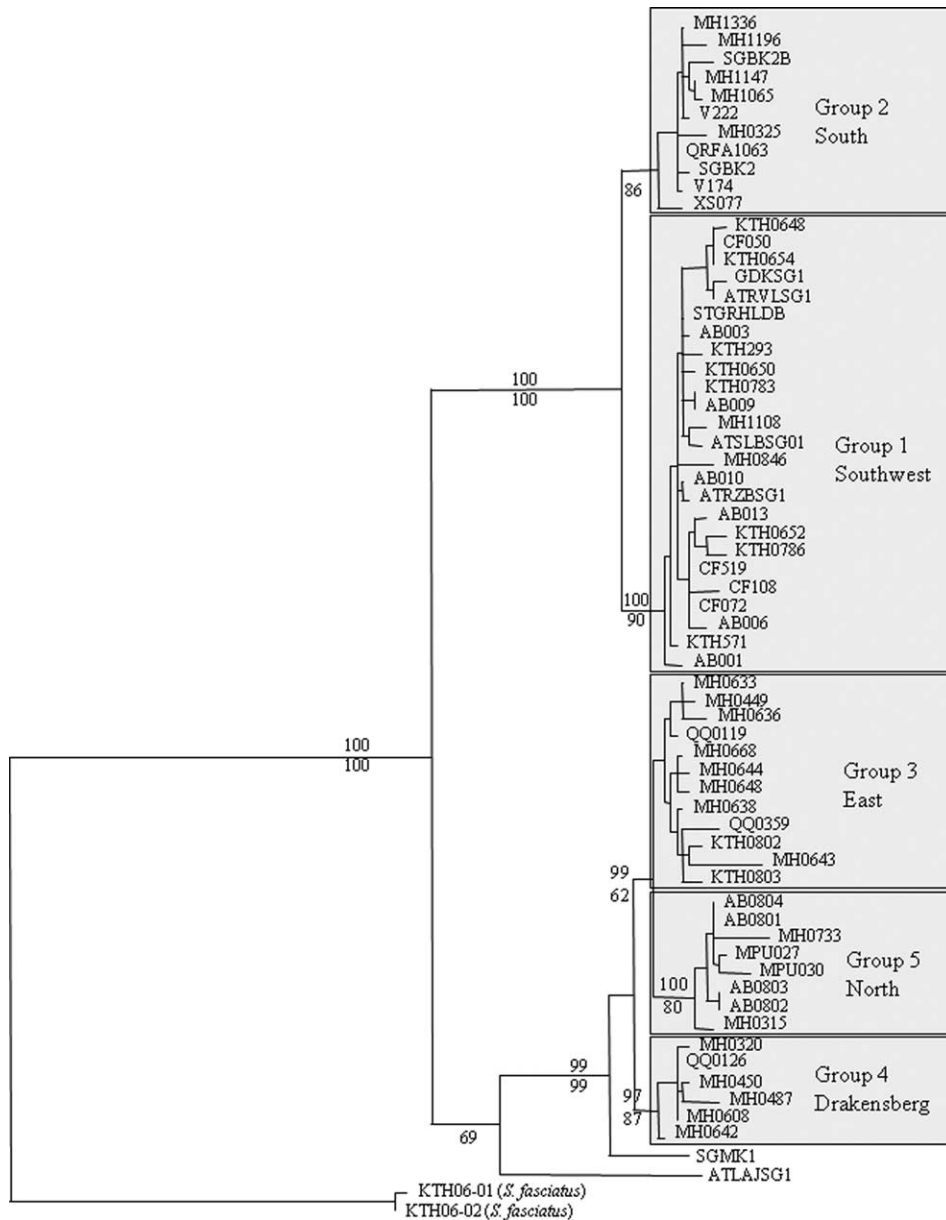


Figure 2. Bayesian majority-rule consensus phylogram for the combined ND2 and 16S data set for *Strongylopus grayii*. Posterior probability values for each node are shown above the branch, and parsimony bootstrap values are shown below. Population groups from SAMOVA (see Fig. 3) are indicated by the grey boxes.

sequences within each phylogenetic clade, median-joining networks were constructed in Network v4.1 (Bandelt *et al.* 1999).

The degree of differentiation among adjacent sampling sites was assessed using a spatial analysis of molecular variance (SAMOVA; Dupanloup *et al.* 2002).

SAMOVA uses the geographical co-ordinates and corresponding sequence data to assign sampling sites *a posteriori* to groups that presumably represent historically cohesive populations. The procedure uses a simulated annealing process to maximise the proportion of total genetic variation between groups of sample sites using traditional F statistics. F_{CT} is the proportion of total genetic variance due to the differences between groups of sample sites; F_{SC} represents the variation between sample sites within groups; while F_{ST} is the genetic variation between sample sites relative to the total sample (Excoffier *et al.* 1992; Dupanloup *et al.* 2002). SAMOVA was run with all possible group structures (K groups) to determine the optimal value for F_{CT} (the maximised proportion of total genetic variance due to differences between groups). Two sample sites (Soutpansberg and Waterberg) were not included in the SAMOVA because each of these comprised a single sample with a divergent position in the phylogenetic analysis.

A coalescent approach was used to investigate the most likely level of historical gene flow between populations identified by SAMOVA, using the isolation with migration model (IMa) and Markov chain Monte Carlo (MCMC) simulations (Hey 2005; Hey and Nielsen, 2007). For this analysis sequence datasets were submitted remotely to the CBSU High-Performance Computing Institute at Cornell University (<http://cbsuapps.tc.cornell.edu/IMa.aspx>). Each simulation run utilised 20 Markov chains, 10 million steps for the burn-in, 10 million steps of the MCMC simulation (sampling every 1 000 generations). A geometric heating scheme was used with $g1 = 0.9$ and $g2 = 0.5$, and 20 chain swap attempts per Metropolis-coupling step. For each population pair, three runs were carried out to examine whether independent simulations converged on the same estimated migration rate.

RESULTS

Phylogenetic Analyses

The basic tree topology was similar for both phylogenetic methods, with differences being restricted to the poorly supported terminal nodes. Parsimony analysis of the 261 informative characters produced 655 trees with a length of 443 steps ($CI = 0.711$; $RI = 0.936$) (results not shown). Parsimony bootstrap support for major nodes varied from 62–100% (Fig. 2). The two parallel Bayesian runs produced identical majority-rule consensus trees. The major nodes were supported with posterior probabilities ≥ 0.95 . Both analyses recovered two main clades: a northern and a southern clade. Within these clades, there were several additional well-supported sub-clades corresponding with geography (Fig. 2). Two samples from the far north (from the Waterberg and Soutpansberg) fell outside these sub-clades, although they were

Table 1. Sample sizes (n), haplotype (h) and nucleotide (π) diversity (with 95% confidence intervals in parentheses) for *Strongylopus grayii* from two phylogenetic clades.

Clade	n	π (95% CI)	h (95% CI)
South	30	0.009 (± 0.005)	0.96 (± 0.019)
North	25	0.009 (± 0.005)	0.98 (± 0.015)
Total	55	0.030 (± 0.015)	0.99 (± 0.007)

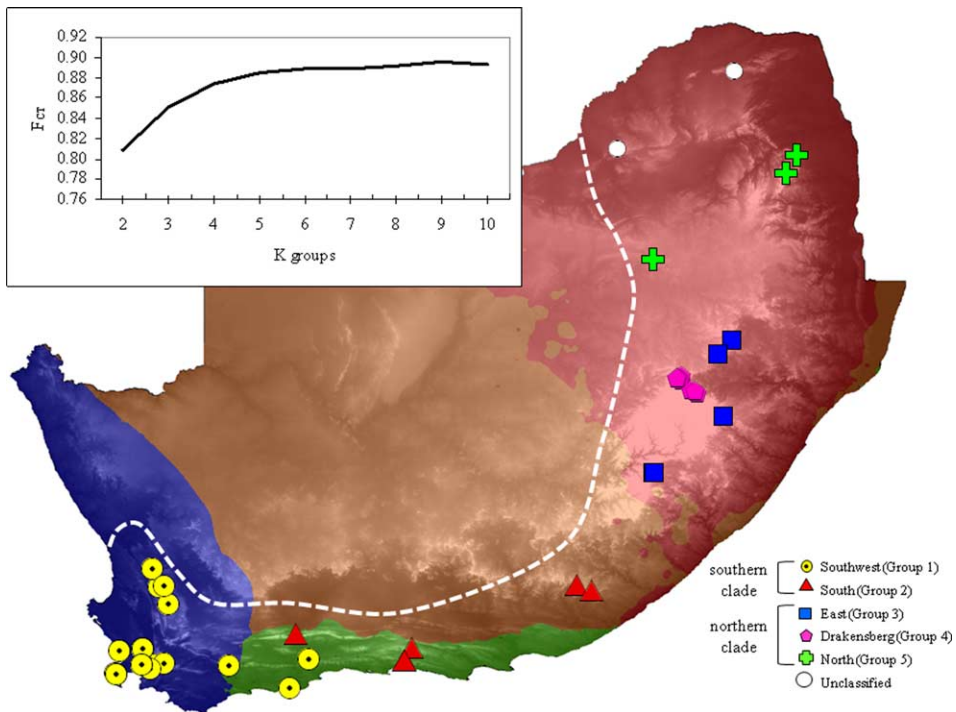


Figure 3. Distribution of sample sites coded to the populations recognised in SAMOVA (yellow/dotted circle = southwest; red/triangle = south; blue/square = east; pink/pentagon = Drakensberg; green/cross = north; white/circle = unclassified). Inset: Relationship between F_{CT} and the number of populations input into the SAMOVA.

both included in the broad northern clade, albeit with lower support. The average uncorrected pairwise sequence divergence between the northern and southern clades was $\sim 7.7\%$ for ND2 and $\sim 0.6\%$ for 16S. Both clades of *S. grayii* had high diversity, with 21 haplotypes in the southern clade (Table 1).

Population Genetic Analyses

Spatial analysis of molecular variance (SAMOVA) indicated that the variance between populations is maximised when the sample sites were partitioned into five groups ($F_{CT}=0.89$), as indicated by the values of F_{CT} reaching a plateau (Fig. 3, inset). When K was larger than five, F_{CT} values continued to increase, but at a negligible rate, essentially due to a corresponding decrease in F_{SC} that would continue until all sampling sites are separate. Hence the most plausible result from SAMOVA that best captures the structure in the data is two southern populations ('Southwest' and 'South'), and three northern populations ('East', 'Drakensberg', and 'North') (Fig. 3). Median-joining networks for each clade were consistent with the SAMOVA populations, with all haplotypes restricted to one or the other populations. These networks also revealed limited uncertainty in relationships

among haplotypes within populations, especially in the southwest (Fig. 4). The pattern in the network reflects the high gene diversity (h), with a large number of haplotypes, none of which are common.

Coalescent analysis of migration rates indicated higher estimates of gene flow between populations within each region (i.e. within southern or within northern populations), than between the regions (Fig. 5). Between regions, gene flow was estimated as very close to zero (Fig. 5a), whereas values were closer to 0.1 between populations within a region (Fig. 5b). However, the posterior density distributions

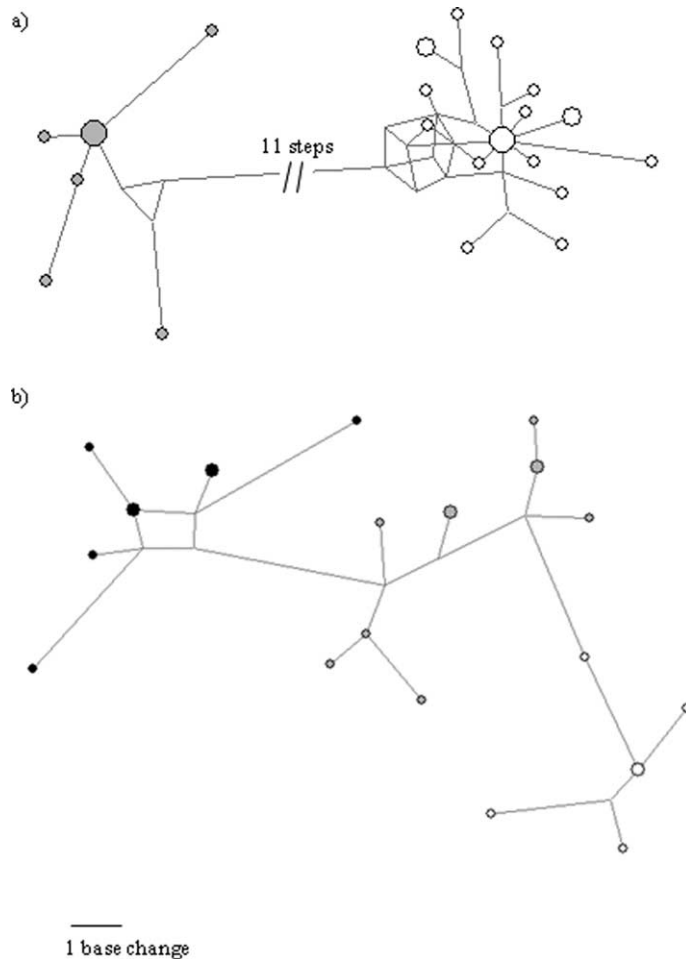


Figure 4. Median-joining haplotype networks for the two phylogenetic clades found for *S. grayii*, (a) southern clade and (b) northern clade. Haplotypes are indicated by the circles, and the circle sizes are proportional to the number of individuals with that haplotype. The lines joining haplotypes are proportional to the number of base changes between the haplotypes (the shortest branches indicate one base change) except where indicated. For each haplotype, the proportion of individuals from each SAMOVA population is shown by shading, (a) southern clade (white = southwest; grey = south), (b) northern clade (white = Drakensberg; grey = east; black = north).

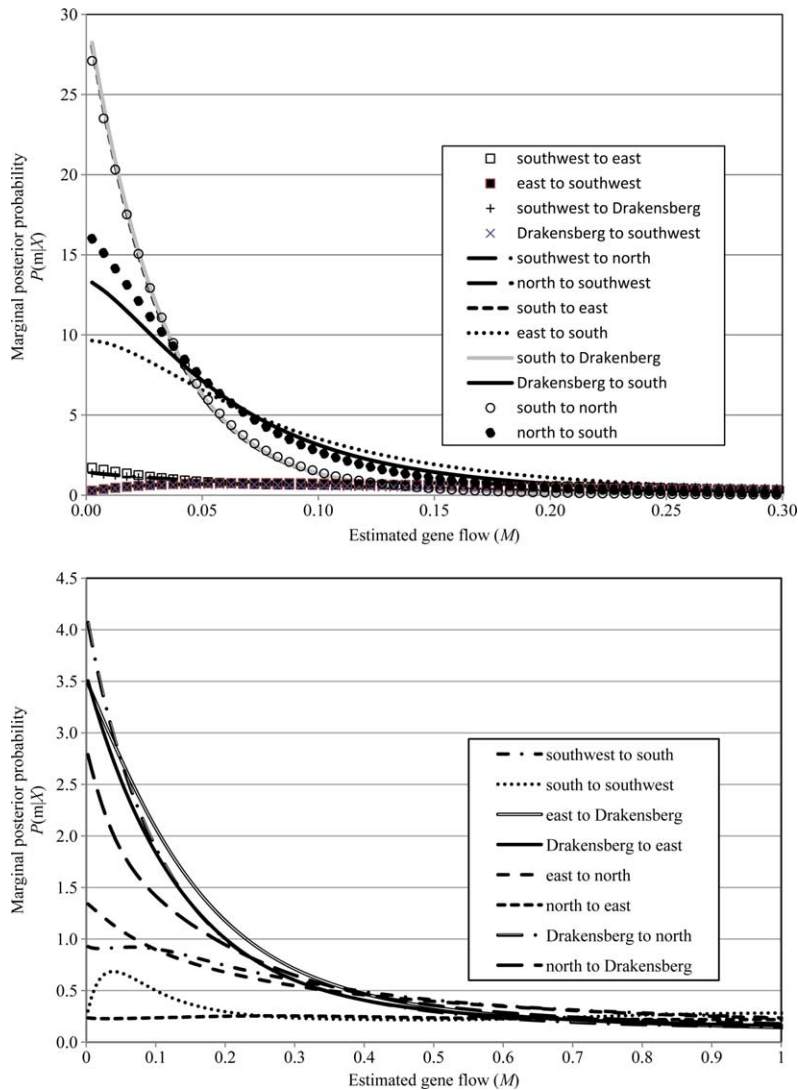


Figure 5. Posterior density distributions for estimates of gene flow (M) between populations of *Strongylopus grayii* for (a) populations between the southern and northern regions, and (b) populations within those regions. Most likely values for gene flow are given by the marginal posterior probability ($P(m|X)$). Note that the scales differ between the figures. Populations are named as in Fig. 1, and the direction of gene flow is indicated in the legend.

suggest the confidence around these estimates is large, and in some cases the function is essentially flat (e.g. east to southwest, Fig. 5a; north to east, Fig. 5b) making some inferences tenuous. Regardless, the estimates for the south + east and south + Drakensberg populations (Fig. 5a), indicated that gene flow is most likely <0.10 between the two regions. In contrast, gene flow between populations within each region is ≥ 0.10 .

DISCUSSION

The mtDNA results from this study show significant genetic structuring across the distribution range of *Strongylopus grayii* in South Africa. Two well-supported clades are geographically partitioned, with one found in the north and the other restricted to the south (Fig. 3). The geographic distribution of these clades roughly corresponds with the summer and the winter + aseasonal rainfall zones (see Chase & Meadows 2007) and gene flow is lacking between these two regions (Fig. 5). Two individuals (from Waterberg and Soutpansberg) are allied to the northern clade but were particularly divergent and could not be placed with confidence in that clade (Fig. 2). Further fine-scale sampling could potentially reveal additional phylogenetic lineages, especially considering the discovery of these very divergent haplotypes. Overall, these two clades represent a reasonably deep divergence (on the order of approximately 8% sequence divergence for ND2), which is comparable to the range of values found for other recognised amphibian sister species (from 6–17%; e.g. Macey *et al.* 1998, 2001; Loader *et al.* 2006; Blackburn 2008). This seems to suggest that there are two cryptic taxa within what is currently recognised as a single species. The divergent individuals, from Waterberg and Soutpansberg cannot be fully assessed here, but an investigation into the possibility of additional cryptic taxa is clearly warranted.

Geographic Patterns

Within each clade, spatial analysis of molecular variance identified several populations, consistent with patterns in the phylogenetic tree (Fig. 2) and the network (Fig. 4), whereby each main clade could be subdivided into populations, three in the north and two in the south. Within the southern clade, the southwest population ranges primarily, but not exclusively, within the winter rainfall zone. The spanning of more than one rainfall regime suggests that rainfall seasonality (winter vs. aseasonal) is not a historical barrier to gene flow as hypothesised. These rainfall zones are geographically adjacent (Chase & Meadows 2007) and temporally overlapping. In the southwest population, frogs have the opportunity to breed in the winter (within the winter rainfall zone) or at any time of the year that is wet (within the aseasonal rainfall zone). Thus, gene flow may not be hampered across these zones, resulting in a single population (southwest) that transcends two rainfall regimes.

The coalescent analysis points to limited gene flow between the southwest and south populations. Because the current rainfall regime does not delimit these two populations, there may be some other factor defining current population structure and limiting dispersal. Two factors not considered in our original hypothesis are the greatly extended coastal plain and the eastward expansion of the winter rainfall zone during the last glacial maximum (Chase & Meadows 2007). The pattern we observe today is most likely a result of historical factors such as these. The present distribution of the southwest clade is suggestive of a clade that has been constricted from a previously enlarged coastal distribution (up to 200 km southwards onto the presently inundated Agulhas Bank) which was forced northward as the sea level rose, and has stalled against a more xeric barrier to the north. Among the three northern

populations, it appears that gene flow is slightly higher (Fig. 5), but still low enough for population genetic structure to be evident.

Overall the pattern of genetic structuring does not favour current rainfall seasonality as a driver of genetic structure in the south, but it is possible that a temporal barrier to gene flow is in place between the two larger regions. In this scenario, gene flow is restricted between northern frogs (breeding in summer) and southern frogs (breeding mainly in winter and autumn). In southern Africa, the only other documented example of genetic structuring as a result of rainfall seasonality is that of *Xenopus laevis* (Measey & Channing 2003), although the situation may be more common than presently understood. In fact, the particular genetic pattern observed for *S. grayii* is reflected in the distribution of other frogs of South Africa (Alexander *et al.* 2004), with many species distributed either north or south of this break in rainfall seasonality. It should also be noted that a number of other taxa from the Cape region of South Africa show similar geographic patterns, sequence divergences, and depth in the phylogenies. These studies generally conclude that environmental changes in the late Pliocene have driven divergence of major clades (e.g. Tolley *et al.* 2006; Price *et al.* 2007; Swart *et al.* 2009) and there is an emerging synthesis of common processes that have influenced regional diversity and biogeographic patterns.

Taxonomic Implications

The large divergence between the southern and northern clades within *S. grayii* is comparable to the level of divergence found between other amphibian species (Turner *et al.* 2004; Vences *et al.* 2005; Measey *et al.* 2007; Channing & Schmitz 2008; Turner & Channing 2008; Blackburn 2009). This, coupled to the spatio-temporal reproductive isolation inferred from the lack of gene flow across the rainfall zones raises the question of whether there is enough evidence to distinguish these clades as separate species. Whilst the number of criteria for species definitions is continually growing, limited attention has been given to methodically testing species boundaries (Wiens 1999; Sites & Marshall 2003). Recent increase in the use of molecular genetic markers in phylogenetic studies has led to the development of species concepts based solely on genetic differences (Crandall *et al.* 2000). While genetic differences can provide useful insight into the discovery of possible cryptic species, it is dangerous to rely on molecular markers alone to delimit species, especially if only using mtDNA (Hoelzer 1997; Sites & Crandall 1997). Discordance may result between the gene tree and the species tree due to incomplete lineage sorting (Hey & Machado 2003). The gene genealogy also reflects the action of selection rather than the species history (Knowles 2004). Therefore, before the *S. grayii* clades can be identified as distinct species, additional data need to be collected. Specifically, increasing the number of samples from intermediate areas would confirm that the observed structuring is not due to the limited coverage of this study. The advertisement calls of frogs from the southern and northern clades need to be recorded and analysed for any slight differences that could indicate a method of pre-reproductive isolation. Morphological data for frogs from southern and northern clades should also be analysed, thus confirming (or rejecting) the possibility that these clades reflect true cryptic species. In addition, further attention should be given to the divergent lineages in the

Waterberg and Soutpansberg, and how these fit into the larger context of the genus. Our data were extremely limited, but at this stage do point to additional cryptic lineages in those regions.

Conclusions

The identification of genetically distinct clades within *S. grayii*, whether separate species or not, requires reconsideration of the management of this species from a conservation perspective (Crandall *et al.* 2000). In amphibians, post-zygotic isolation correlates with pairwise genetic distance (Sasa *et al.* 1998). Given the extent of the genetic structuring present in *S. grayii*, it is possible that frogs from the southern and northern clades are unable to interbreed due to temporal constraints on breeding season. Assuming this is true, the two clades would qualify, at a minimum, as Evolutionary Significant Units, being reproductively isolated and contributing substantially to the genetic diversity of the species as a whole (Waples 1991).

ACKNOWLEDGEMENTS

We would like to thank the South African National Biodiversity Institute (SANBI) and the WWF-Table Mountain Fund for providing the funding for this study. AB was also supported through SANBI short-term student funding to conduct the laboratory component of this study. We are grateful for the assistance in the field from Marius Burger, Kate Henderson, John Measey, and Andrew Turner, and to Tony Verboom who provided valuable comments on early drafts of this work and guidance to AB during her BSc work at the University of Cape Town.

REFERENCES

- ALEXANDER, G.J., J.A. HARRISON, D.H. FAIRBANKS & R.A. NAVARRO. 2004. Biogeography of the frogs of South Africa, Lesotho and Swaziland. Pp. 31–47. *In* L.R. Minter, M. Burger, J.A. Harrison, H.H. Braack, P.J. Bishop & D. Kloepfer (Eds.), *Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland*. SI/MAB Series #9. Smithsonian Institution, Washington, DC, USA.
- AVISE, J.C. 2000. *Phylogeography: The History and Formation of Species*. Harvard University Press, Cambridge, MA, USA.
- BANDELT, H.J., P. FORSTER & A. RÖHL. 1999. Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* 16: 37–48.
- BEHEREGARAY, L.B. 2008. Twenty years of phylogeography: the state of the field and challenges for the Southern Hemisphere. *Mol. Ecol.* 17: 3754–3774.
- BEHEREGARAY, L.B. & A. CACCONI. 2007. Cryptic biodiversity in a changing world. *J. Biol.* 6: 1–5.
- BICKFORD, D., D.J. LOHMAN, N.S. SODHI, P.K. NG, R. MEIER, K. WINKER, K.K. INGRAM & I. DAS. 2007. Cryptic species as a window on diversity and conservation. *Trends Ecol. Evol.* 22: 391–392.
- BLACKBURN, D.C. 2008. Biogeography and evolution of body size and life history of African frogs: phylogeny of squeakers (*Arthroleptis*) and long-fingered frogs (*Cardioglossa*) estimated from mitochondrial data. *Mol. Phylogenet. Evol.* 49: 806–826.
- BLACKBURN, D.C. 2009. Description and phylogenetic relationships of two new species of miniature *Arthroleptis* (Anura: Arthroleptidae) from the Eastern Arc Mountains of Tanzania. *Breviora* 517: 1–17.
- BOSSUYT, F., R.M. BROWN, D.M. HILLIS, D.C. CANNATELLA & M.C. MILINKOVITCH. 2006. Phylogeny and biogeography of a cosmopolitan frog radiation: Late Cretaceous diversification resulted in continent scale endemism in the family Ranidae. *Syst. Biol.* 55: 579–594.

- BUCKLEY, D. 2009. Toward an organismal, integrative, and iterative phylogeography. *BioEssays* 31: 784–793.
- CHANNING, A. 1986. A new species of *Strongylopus* Tschudi from Namaqualand, Cape Province, South Africa (Anura: Ranidae). *Ann. Cape Prov. (Nat. Hist.)* 16: 127–135.
- CHANNING, A. 2004. *Strongylopus grayii* (Smith 1849). Pp. 311–312. In L.R. Minter, M. Burger, J.A. Harrison, H.H. Braack, P.J. Bishop & D. Kloepfer (Eds.), *Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland*. SI/MAB Series #9. Smithsonian Institution, Washington, DC, USA.
- CHANNING, A. & T.R.B. DAVENPORT. 2002. A new stream frog from Tanzania (Anura: Ranidae: *Strongylopus*). *Afr. J. Herpetol.* 51: 135–142.
- CHANNING, A. & A. SCHMITZ. 2008. Hiding in plain sight: another cryptic dainty frog from the highlands of Kenya (Anura: Pyxicephalidae: *Cacosternum*). *Afr. J. Herpetol.* 57: 75–84.
- CHASE, B.M. & M.E. MEADOWS. 2007. Late Quaternary dynamics of southern Africa's winter-rainfall zone. *Earth-Sci. Rev.* 84: 103–138.
- CLARKE, B.T. & J.C. POYNTON. 2005. A new species of Stream Frog, Genus *Strongylopus* (Anura: Ranidae) from Mount Kilimanjaro, Tanzania, with comments on a 'northern volcano mountains group' within the genus. *Afr. J. Herpetol.* 54: 53–60.
- CRANDALL, K.A., O.R.P. BININDA-EMONDS, G.M. MACE & R.K. WAYNE. 2000. Considering evolutionary process in conservation biology. *Trends Ecol. Evol.* 15: 290–295.
- CUNNINGHAM, M. & M.I. CHERRY. 2004. Molecular systematics of African 20-chromosome toads (Anura: Bufonidae). *Mol. Phylogenet. Evol.* 32: 671–685.
- DAWOOD, A., A. CHANNING & J.P. BOGART. 2002. A molecular phylogeny of the frog genus *Tomopterna* in Southern Africa: examining species boundaries with mitochondrial 12S rRNA sequence data. *Mol. Phylogenet. Evol.* 22: 407–413.
- DUPANLOUP, I., S. SCHNEIDER & L. EXCOFFIER. 2002. A simulated annealing approach to define the genetic structure of populations. *Mol. Ecol.* 11: 2571–2581.
- DU PREEZ, L. & V. CARRUTHERS. 2009. *A Complete Guide to the Frogs of Southern Africa*. Struik Nature, Cape Town.
- EXCOFFIER, L., P.E. SMOUSE & J.M. QUATTRO. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131: 479–491.
- FOUQUET, A., A. GILLES, M. VENCES, C. MARTY, M. BLANC & N.J. GEMMELL. 2007. Underestimation of species richness in neotropical frogs revealed by mtDNA analyses. *PLoS ONE* 2: e1109.
- FROST, D.R. 2009. Amphibian species of the world: an online reference. Version 5.3 (12 February 2009). Electronic database accessible at <http://research.amnh.org/herpetology/amphibia/>. American Museum of Natural History, New York, USA.
- FROST, D.R., T. GRANT, J. FAIVOVICH, R.H. BAIN, A. HAAS, C.F.B. HADDAD, R.O. DE SÁ, A. CHANNING, M. WILKINSON, S.C. DONNELLAN, C.J. RAXWORTHY, J.A. CAMPBELL, B.L. BLOTTO, P. MOLER, R.C. DREWES, R.A. NUSSBAUM, J.D. LYNCH, D.M. GREEN & W.C. WHEELER. 2006. The amphibian tree of life. *B. Am. Mus. Nat. Hist.* 297: 370pp.
- FROST, D.R., T. GRANT, J. FAIVOVICH, R.H. BAIN, A. HAAS, C.F.B. HADDAD, R.O. DE SÁ, A. CHANNING, M. WILKINSON, S.C. DONNELLAN, C.J. RAXWORTHY, J.A. CAMPBELL, B.L. BLOTTO, P. MOLER, R.C. DREWES, R.A. NUSSBAUM, J.D. LYNCH, D.M. GREEN & W.C. WHEELER. 2008. Is *The Amphibian Tree of Life* really fatally flawed? *Cladistics* 24: 385–395.
- HEWITT, J. 1933. On new and rare frogs from Chirinda Forest. *Occas. Pap. Nat. Mus. S. R.* 6: 12–13 plate I.
- HEY, J. 2005. On the number of new world founders: a population genetic portrait of the peopling of the Americas. *PLOS Biology* 3: e193.
- HEY, J. & C.A. MACHADO. 2003. The study of structured populations – new hope for a difficult and divided science. *Nat. Rev. Gen.* 4: 535–543.
- HEY, J. & R. NIELSEN. 2007. Integration within the Felsenstein equation for improved Markov chain Monte Carlo methods in population genetics. *Proc. Natl. Acad. Sci. USA* 104: 2785–2790.
- HILLIS, D.M. 2007. Constraints in naming parts of the tree of life. *Mol. Phylogenet. Evol.* 42: 331–338.
- HIPP, A.L., J.C. HALL & K.J. SYTSMA. 2004. Congruence versus phylogenetic accuracy: revisiting the incongruence length difference test. *Syst. Biol.* 53: 81–89.
- HOELZER, G.A. 1997. Inferring phylogenies from mtDNA variation: Mitochondrial-Gene trees versus Nuclear-Gene Trees revisited. *Evolution* 51: 622–626.

- HOLLAND, B.R., K.T. HUBER, V. MOULTON & P. LOCKHART. 2004. Using consensus networks to visualise contradictory evidence for species phylogeny. *Mol. Biol. Evol.* 21: 1459–1461.
- HUELSENBECK, J.P. & F. RONQUIST. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755.
- JACOBSEN, N.H.G. 1989. A herpetological survey of the Transvaal. Unpubl. Ph.D. thesis, University of Natal, Pietermaritzburg.
- KNOWLES, L.L. 2004. The burgeoning field of statistical phylogeography. *J. Evolution Biol.* 17: 1–10.
- KÖHLER, J., D.R. VIEITES, R.M. BONETT, F.H. GARCÍA, F. GLAW, D. STEINKE & M. VENCES. 2005. New amphibians and global conservation: a boost in species discoveries in a highly endangered vertebrate group. *BioScience* 55: 693–696.
- KUMAR, S., K. TAMURA, I.B. JAKOBSEN & M. NEI 2001. MEGA version 2.1. Molecular Evolutionary Genetics Analysis software. Arizona State University, Tempe, Arizona, USA.
- LOADER, S.P., A. CHANNING, M. MENEGON & T.R.B. DAVENPORT. 2006. A new species of *Probreviceps* (Amphibia: Anura) from the Eastern Arc Mountains, Tanzania. *Zootaxa* 1237: 45–60.
- MACEY, J.R., J.A. SCHULTE II, A. LARSON, Z. FANG, Y. WANG, B.S. TUNIYEV & T.J. PAPENFUSS. 1998. Phylogenetic relationships of toads in the *Bufo bufo* species group from the eastern escarpment of the Tibetan Plateau: a case of vicariance and dispersal. *Mol. Phylogenet. Evol.* 9: 80–87.
- MACEY, R.J., J.L. STRASBURG, J.A. BRISSON, V.T. VREDENBURG, M. JENNINGS & A. LARSON. 2001. Molecular phylogenetics of western North American frogs of the *Rana boylei* species group. *Mol. Phylogenet. Evol.* 19: 131–143.
- MADDISON, W.P. & D.R. MADDISON 1992. MacClade: analysis of phylogeny and character evolution, version 4.0. Sinauer Associates, Sutherland, Massachusetts, USA.
- MEASEY, G.J. & A. CHANNING. 2003. Phylogeography of the genus *Xenopus* in Southern Africa. *Amphibia-Reptilia* 24: 321–330.
- MEASEY, G.J., M. VENCES, R.C. DREWES, Y. CHIARI, M. MELO & B. BOURLES. 2007. Freshwater paths across the ocean: molecular phylogeny of the frog *Ptychadena newtoni*. *J. Biogeogr.* 34: 7–20.
- MINTER, L.R., M. BURGER, J.A. HARRISON, H.H. BRAACK, P.J. BISHOP & D. KLOEPFER (Eds.) 2004. Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland. SI/MAB Series #9. Smithsonian Institution, Washington, DC, USA.
- NEI, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.
- PALUMBI S. 1991. The simple fool's guide to PCR. Biol101. Version 2. University of Hawaii, Honolulu, Hawaii.
- PICKERSGILL, M. 2007. Frog Search: Results of Expeditions to Southern and Eastern Africa. Edition Chimaira, Frankfurt am Main.
- POSADA, D. & K.A. CRANDALL. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817–818.
- POSADA, D. & K.A. CRANDALL. 2001. Intraspecific gene genealogies: trees grafting into networks. *Trends Ecol. Evol.* 16: 37–45.
- POYNTON, J.C. 2004. Stream frogs in Tanzania (Ranidae: *Strongylopus*): the case of *S. merumontanus* and *S. fuelleborni*. *Afr. J. Herpetol.* 53: 29–34.
- PRICE, B.W., N.P. BARKER & M.H. VILLET. 2007. Patterns and processes underlying evolutionary significant units in the *Platypleura stridula* L. species complex (Hemiptera: Cicadidae) in the Cape Floristic Region, South Africa. *Mol. Ecol.* 16: 2574–2588.
- RAMBAUT, A. & A.J. DRUMMOND. 2007. Tracer v1.4. Available from <http://beast.bio.ed.ac.uk/Tracer>
- RONQUIST, F. & J.P. HUELSENBECK. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.
- SASA, M.M., P.T. CHIPPENDALE & N.A. JOHNSON. 1998. Patterns of postzygotic isolation in frogs. *Evolution* 52: 1811–1820.
- SCHNEIDER, S., D. ROESSLI & L. EXCOFFIER. 2000. Arlequin version 2.000: a software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.
- SITES, J.W. & K.A. CRANDALL. 1997. Testing species boundaries in biodiversity studies. *Conserv. Biol.* 11: 1289–1297.
- SITES, J.W. & J.C. MARSHALL. 2003. Delimiting species: a Renaissance issue in systematic biology. *Trends Ecol. Evol.* 18: 462–470.
- STUART, B.L., R.F. INGER & H.K. VORIS. 2006. High level of cryptic species diversity revealed by sympatric lineages of Southeast Asian forest frogs. *Biol. Letters* 2: 470–474.

- SWART, B.L., K.A. TOLLEY & C.A. MATTHEE. 2009. Climate change drives speciation in the Southern Rock Agama (*Agama atra*) in the Cape Floristic Region, South Africa. *J. Biogeogr.* 36: 78–87.
- Swofford, D.L. 2002. PAUP* phylogenetic analysis using parsimony (*and other methods), version 4.0b.10. Sinauer Associates, Sutherland, Massachusetts.
- TAJIMA, F. 1983. Evolutionary relationship of DNA sequences in finite populations. *Genetics* 105: 437–460.
- TARRANT, J., M.J. CUNNINGHAM & L.H. DU PREEZ. 2008. Maluti mystery: a systematic review of *Amietia vertebralis* (Hewitt, 1927) and *Strongylopus hymenopus* (Boulenger, 1920) (Anura: Pyxicephalidae). *Zootaxa* 1962: 33–48.
- THOMPSON, J.D., T.J. GIBSON, F. PLEWNIAL, F. JEANMOUGIN & D.G. HIGGINS. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 24: 4876–4882.
- TOLLEY, K.A., M. BURGER, A.A. TURNER & C.A. MATTHEE. 2006. Biogeographic patterns and phylogeography of dwarf chameleons (*Bradypodion*) in an African biodiversity hotspot. *Mol. Ecol.* 15: 781–793.
- TURNER, A.A. & A. CHANNING. 2008. A new species of *Arthroleptella* Hewitt, 1926 (Anura: Pyxicephalidae) from the Klein Swartberg Mountain, Caledon, South Africa. *Afr. J. Herpetol.* 57: 1–12.
- TURNER, A.A., A.L. DE VILLIERS, A. DAWOOD & A. CHANNING. 2004. A new species of *Arthroleptella* Hewitt, 1926 (Anura: Ranidae) from the Groot Winterhoek mountains of the Western Cape Province, South Africa. *Afr. J. Herpetol.* 53: 1–12.
- VAN DER MEIJDEN, A., M. VENCES, S. HOEGG & A. MEYER. 2005. A previously unrecognized radiation of ranid frogs in Southern Africa revealed by nuclear and mitochondrial DNA sequences. *Mol. Phylogenet. Evol.* 37: 674–685.
- VENCES, M., M. THOMAS, A. VAN DER MEIJDEN, Y. CHIARI & D.R. VIEITES. 2005. Comparative performance of the rRNA gene in DNA barcoding of amphibians. *Front. Zool.* 2: 5.
- VIEITES, D.R., K.C. WOLLENBERG, F. ANDREONE, J. KÖHLER, F. GLAW & M. VENCES. 2009. Vast underestimation of Madagascar's biodiversity evidenced by an integrated amphibian inventory. *Proc. Natl. A. Sci. USA* 106: 8267–8272.
- WAPLES, R.S. 1991. Pacific salmon, *Oncorhynchus* spp., and the definition of 'species' under the endangered species act. *Mar. Fish. Rev.* 53: 11–22.
- WIENS, J.J. 1999. Polymorphism in systematics and comparative biology. *Annu. Rev. Ecol. Syst.* 30: 327–362.

Received: 23 December 2009; Final acceptance: 3 March 2010