Isolation and high genetic diversity in dwarf mountain toads (*Capensibufo*) from South Africa

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Traditional models of amphibian dispersal and gene flow point to low dispersal and high philopatry. In recent years, this traditional view has been challenged and it appears that no general model holds across taxa. Conservation of amphibians cannot be addressed on an over-arching scale, but must come on a case-by-case basis, especially for range-restricted species where information on gene flow and migration must be incorporated into conservation efforts. The only two members of the genus *Capensibufo* Grandison, 1980 (Anura: Bufonidae) are range restricted small bufonids, with distributions limited to montane areas in South Africa. Using a Bayesian analysis of two mitochondrial markers (16S and ND2), we examined the genetic patterns in *Capensibufo rosei* and *Capensibufo tradouwi* in order to understand both taxonomic and geographic boundaries. These species were not monophyletic, and demonstrate no clear taxonomic boundaries. Instead, the genus is extremely diverse genetically, with distinct lineages confined to isolated mountains that represent geographic boundaries. In addition, bioclimatic modelling using MAXENT and scenarios of climatic conditions at both the present and last glacial maximum suggest multiple bioclimatic and physical barriers to gene flow at present and in the past. We conclude that members of the genus have very low vagility, that current taxonomic boundaries are inadequate, and that strong geographic structuring has undoubtedly contributed to genetic diversity at the species level, rather than the population level. © 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2010, **100**, 822–834.

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INTRODUCTION

Traditionally, amphibians are considered to be poor dispersers (Blaustein, Wake & Sousa, 1994), and strongly philopatric, resulting in strong genetic structure (Avise, 2000), with speciation mainly through vicariance (Vences & Wake, 2007). This traditional view is backed up by numerous ecological studies on amphibians, which show small home ranges (e.g. Fellers & Kleeman, 2007), short migrational distances (Semlitsch, 2008), and requirements for continuous habitat (Funk *et al.*, 2005). An alternative view comes from a series of studies that have turned the traditional view on its head. Some amphibians are shown to be very strong dispersers that can exploit new habitats (Phillips *et al.*, 2006a), and conform to larger metapopulation models (Marsh & Trenham, 2000). Some species are genetically uniform over large areas (Gower *et al.*, 2007; Hu *et al.*, 2007), and individuals migrate over long distances (Smith & Green, 2005). Dispersal across 'strong barriers' seems to be achievable, including movement over salt water

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(e.g. Vences *et al.*, 2003; Measey *et al.*, 2007b) and dry, inhospitable habitats (Measey *et al.*, 2007a; Kuchta *et al.*, 2009). Clearly there is no generally applicable model for amphibian dispersal. Underlying this, our understanding of amphibian biology is inadequate for the majority of species, despite the fact that amphibians make up a large proportion of the global vertebrate fauna.

In recent years, population genetic studies have improved our knowledge of amphibian demographic patterns, together with a plethora of methodologies that can inform conservationists (Jehle & Arntzen, 2002; Beebee, 2005). Among anuran amphibians, toads (family Bufonidae) have gained a reputation for high dispersal abilities (Phillips *et al.*, 2006a; Pramuk *et al.*, 2008). However, this is clearly not universal within the family, which contains many range-restricted species (e.g. Menegon, Salvidio & Loader, 2004; Menegon *et al.*, 2007; Measey & Tolley, in press; see also Van Bocxlaer *et al.*, 2010). In particular, there are several genera of African dwarf toads (e.g. *Nectophrynoides* and *Mertensophryne*) that are range restricted within montane habitats, but have varying life-history traits (see Menegon *et al.*, 2004; Ngwava, Malonza & Measey, 2009; Van Bocxlaer *et al.*, 2010). Although there have been increasing numbers of genetic studies showing that large bufonids are wide-ranging animals with the potential for high gene flow, very little information is available on the small, range-restricted members of the family, the conservation status of which is of increasing concern.

The genus *Capensibufo* Grandison, 1980 (Anura: Bufonidae) is one such group of toads. The genus contains only two species, which are confined to isolated, high-altitude mountain plateaus in the Cape Fold Mountains (CFMs) of South Africa. Prior to the erection of the genus *Capensibufo*, they were thought to be related to species now in the genera *Mertensophryne* and *Vandijkophrynus* (see Tandy & Keith, 1972; Frost *et al.*, 2006), and doubt concerning generic monophyly continued for more than 20 years (Graybeal & Cannatella, 1995). Only recently has molecular evidence been used to confirm that *Capensibufo* are indeed each other's closest relatives (Cunningham & Cherry, 2004). They are small frogs (*c.* 2–4 cm in length) that breed in ephemeral pools formed by winter rainfall, usually in or adjoining perennial seepages (Grandison, 1980; de Villiers, 2004b). These pools usually persist throughout winter, and during episodic rainfall they can overflow into small, ephemeral streams. Thus, there is a potential for limited dispersal via eggs or tadpoles washed into these small streams, but this would only occur during a small window of opportunity that coincides with strong bouts of rainfall during early life-history stages. Essentially, gene flow is expected to be minimal, conforming to the low estimated probabilities of the optimal range-expansion phenotype [an index made up of seven life-history traits that were found to correlate with the global colonization and radiation of toads (see Van Bocxlaer *et al.*, 2010)].

For a small toad, the distribution of each species of *Capensibufo* is surprisingly large [*Capensibufo rosei* (Hewitt, 1926) 6500 km^2; *Capensibufo tradouwi* (Hewitt, 1926) 8500 km^2], as estimated by totalling the area of quarter-degree grid cells in which there is any recorded occurrence (Boycott, 2004; de Villiers, 2004a; de Villiers, 2004b). Despite this, the actual area they occupy is much smaller, being almost totally confined to 'fynbos' (a heathland vegetation type that is endemic to the Cape of Africa, see Mucina & Rutherford, 2006) montane plateaus. They are allopatric, with *C. rosei* in the south-western Cape and *C. tradouwi* to the north and the east (Fig. 1). Grandison (1980) hypothesized that the Breede River valley and the Nuwe Kloof correspond to this line of allopatric separation, and form a barrier to dispersal. However, the topography is complex and there are a number of other valleys in the region that could be strong barriers (see Fig. 1). Thus, patchy distributions on montane plateaus are separated by large lowland gaps with unsuitable habitat. The question then arises: why would only one or two valleys separate the two species when these toads are expected to be limited in their dispersal ability through every valley?

Few amphibians in South Africa have this type of strictly montane lifestyle. Moss frogs (genus *Arthroleptella*) are fairly comparable, being of small body size, confined to montane seepages, and generally winter breeders (Channing, 2004). Despite little overall morphological differences (Dawood & Channing, 2000), *Arthroleptella* show high genetic distances between adjacent mountains (Turner & Channing, 2008), essentially producing a one mountain–one species model. In contrast, other CFM amphibians display low genetic structure, especially when their distribution covers both mountains and lowlands (Measey & Channing, 2003; Measey & Tolley, in press; Tolley *et al.*, 2010). Overall, the expectation is that the specialized lifestyle of these strict montane bufonid endemics makes these species prone to poor dispersal, resulting in strong genetic structuring.

In order to understand the evolutionary history and the potential for gene flow between mountains, we utilized phylogenetic analyses of two mitochondrial markers (ND2 and 16S). We hypothesized that if the valleys proposed by Grandison (1980) were significant barriers between species, then individuals from either side of this barrier would form reciprocally monophyletic clades. We also hypothesized that gene flow within species (between mountains) would be limited,
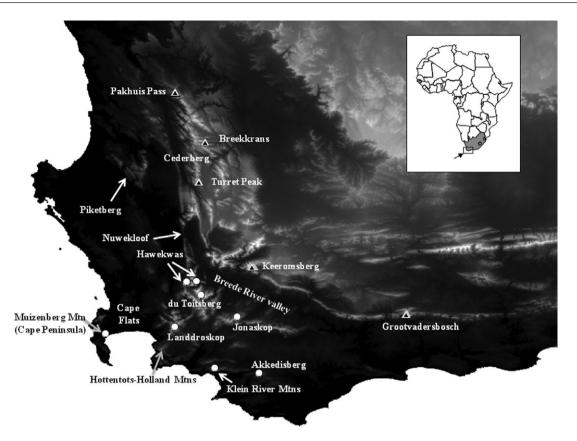
Figure 1. Sampling localities of *Capensibufo rosei* (circles) and *Capensibufo tradouwi* (triangles). Other major geographic features discussed in the text are labelled.

and this would be reflected in the phylogenetic tree. We employed bioclimatic models to examine the level of fragmentation predicted in the present-day distribution of these species, and whether environmental factors dating back to the last glacial maximum (LGM) might be informative to the processes that delimit gene flow in this genus.

MATERIAL AND METHODS

PHYLOGENETICS

Toe clips or tadpole tail tips were taken from 30 *Capensibufo* collected between 2000 and 2005 from 13 localities (Fig. 1), and stored in 70–99% ethanol. Total genomic DNA was extracted using the Qiagen DNeasy kit, and portions of two mitochondrial markers (ND2 543 bp and 16S 644 bp) were sequenced. Amplifications were carried out in 25-µL reaction volumes containing 2 µL of extract (*c.* 25 ng µL^{-1}), 0.25 µM of each primer, 0.2 mM dNTPs, 2.5 mM MgCl$_2$, 1 × thermophilic Buffer (50 mM KCl, 10 mM Tris-HCl, pH 9), and 0.25 U Super-Therm Taq polymerase, using primers vMet and vTrp for ND2 (Cunningham & Cherry, 2004), and L2510 and H3080 for 16S (Palumbi *et al*., 1991). The PCR profile was

95 °C for 1 min, followed by 35 cycles of 35 s at 95 °C, 30 s at 50–55 °C, and 1 min at 72 °C, with a final extension at 72 °C for 30 s. PCR products were visualised on 0.8% agarose gels containing ethidium bromide, and cycle sequenced using a fluorescently labelled dye-terminator kit (ABI, Foster City, CA, USA), purified with Sephadex spin columns and analysed on an Applied Biosystems 3100 genetic analyser. Sequence alignment was carried out in ClustalX using default parameters, and sequences were checked in MacClade 4.0 (Maddison & Maddison, 2000). Two out-group taxa (*Vandijkophrynus angusticeps* and *Vandijkophrynus gariepensis*) were used, based on their close relationship to this genus (see Frost *et al*., 2006). All sequences were deposited in the European Molecular Biology Laboratory (EMBL) database (FN650110–FN650139 and FN652315–FN652342; sequences for *V. gariepensis* downloaded from GenBank, AF463792 and AF220902).

Prior to phylogenetic analysis, Modeltest 3.6 (Posada & Crandall, 1998) was run for each marker and for the combined data set to investigate the evolutionary model that best fit the data. The LRT and AIC tests specified either HKY + G or TrN + G,

respectively, for ND2, TrN + G or TVM + I for 16S, and HKY + I + G or GTR + I + G for the combined data set. The partition homogeneity test indicated no conflict between the two markers ($P = 0.69$), so the markers were analysed to produce a single phylogeny. Bayesian inference was run using MrBayes 3.1.0 (Huelsenbeck & Ronquist, 2001). The phylogeny was estimated with two data partitions (two rate categories + gamma for ND2, six rate categories + gamma for 16S), and uniform priors for all parameters, with each partition allowed to run independently. The Bayesian run was also set up as above, but with four partitions: three partitions for ND2: first, second, and third codons separately, with the 16S run in a single partition. The Markov chain Monte Carlo (MCMC) was run twice in parallel for 10 million generations, sampling trees every 1000 generations. For all runs, the first 3 million generations (3000 trees) were removed as burn-in before constructing a 50% majority-rule tree. Burn-in was determined by examining the stationarity of log-likelihood tree scores and the standard deviation of split frequencies, with an effective sample size (ESS) > 200 for all parameters. Nodes with posterior probability ≥ 0.95 were considered to be supported.

In addition, an unweighted parsimony analysis with heuristic search (TBR branch swapping) was performed using 1000 random-addition replicates in PAUP*4.0b10 (Swofford, 2002). Confidence in the nodes was assessed by 1000 bootstrap replicates, each with 100 random-addition replicates. A maximum likelihood (ML) search was run in GARLI (Zwickl, 2006), using the simple two-parameter model indicated by Modeltest for the combined data set, with all parameters estimated, and a random starting tree. This analysis was run three times to ensure that independent ML searches produced the same topologies. Nodes with a bootstrap value of ≥ 75% were considered to be supported in both these analyses.

Net sequence divergences (p distances) between species (corrected for within species divergence) were estimated in MEGA 4.0 (Tamura *et al.*, 2007). Net divergences between sample sites (corrected for within-site divergence) were also estimated in order to compare the magnitude of divergence between species with that between sites.

To investigate whether the two species could be considered monophyletic, several alternative backbone constrained topologies were created using MacClade 4.0 (see results for constraints). The set of trees (post burn-in from the Bayesian analysis, and the set of ML bootstrap trees) were then filtered in PAUP according to each constraint. The percentage of trees agreeing with that constraint were then used as an estimate of support associated with that particular node.

BIOCLIMATIC MODELLING

Presence data were taken from the CapeNature database, and filtered to remove all data points without degree/minute/second accuracy. The resulting data set contained 30 *C. rosei* and 65 *C. tradouwi* records. Bioclimatic modelling was carried out with MAXENT 2.3 (Phillips *et al.*, 2006b), which is reliant on presence-only data. Climate data for 1.7×1.7 km cells included monthly precipitation, and mean, minimum, and maximum temperatures (Schulze, 1997). Soil type, ecoregion, and altitude were also included (MacVicar *et al.*, 1977; Soil Classification Working Group, 1991; Low & Rebelo, 1996; Schulze, 1997). We used this initial data set of 51 variables as a training set to select the most relevant variables. The default parameters of MAXENT provided a logistic model, using a random test of 20% for training, 1000 iterations, a convergence threshold of 0.00001, 1000 maximum iterations, and a maximum of 10 000 background points. Variables that did not improve the predictive value according to both jack-knife tests were removed. Winter precipitation (June–August), which had the highest contribution to models in jack-knife tests using both test and training gain, was averaged into a single precipitation category (mean of June–August for each 1.7×1.7 km cell using the ArcMap 9 Spatial Analyst tool). Descriptions of distributions and calculations of Extent and Area of Occurrence (EOO and AOO; see IUCN, 2001) were made using the minimum training presence as the threshold for predicted occurrence. Models produced were evaluated by the highest area under the curve (AUC) statistic and receiver operating characteristic (ROC) plots (see Fielding & Bell, 1997).
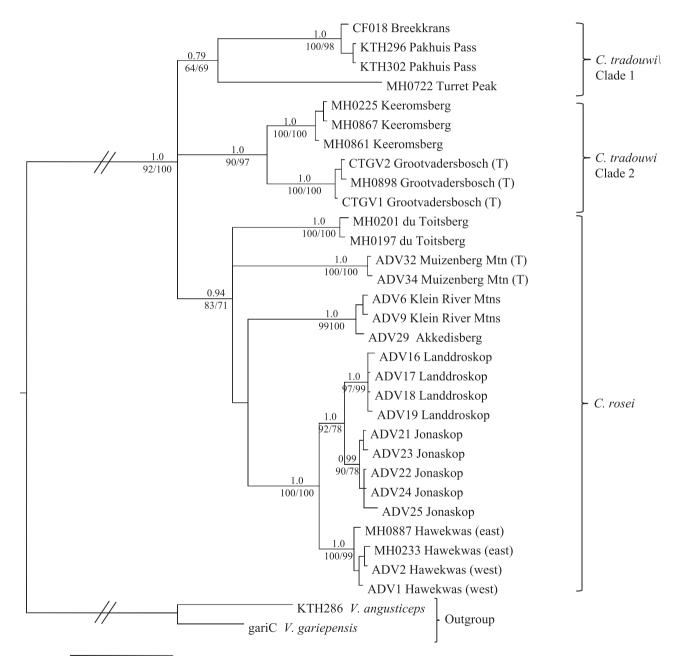
To understand whether historical climate changes may have affected the distribution of these species, the potential climatic space for *Capensibufo* at the LGM was modelled. We used a standard model projection function integrated with MAXENT (Phillips, Anderson & Schapire, 2006b) with the 'palaeoclimatic' equivalents of the variables used in the present-day model. Of the variables in our model, only temperature and rainfall are likely to have changed during the previous 18 Kyr (ignoring the known changes in sea level), so we adjusted the parameters used in the model to theoretical maxima and minima using a recent consensus of palaeoclimatic studies (Chase & Meadows, 2007). Therefore, for the palaeoclimate model, a mean winter rainfall variable (the arithmetic mean of June–August precipitation) was adjusted according to maxima and minima to the LGM (Chase & Meadows, 2007) by simply adding or subtracting to each cell using the ArcMap 9 Spatial Analyst tool.

RESULTS

PHYLOGENETICS

Bayesian, parsimony, and likelihood methods all produced the same basic topology (Fig. 2, Bayesian consensus topology shown). The Bayesian 50% majority rule consensus topologies and associated posterior probabilities did not differ between the runs, nor between the two- and four-partition models (Fig. 2).

Parsimony analysis produced 71 equally parsimonious trees (237 informative sites; tree length = 535; consistency index, CI = 0.686; retention index, RI = 0.858).

Overall, the results produced are not consistent with two monophyletic species. Although *C. rosei* lineages all cluster together with reasonable support, *C. tradouwi* does not. *Capensibufo tradouwi* consists of two divergent clades corresponding with mountain

Figure 2. Bayesian consensus phylogram for *Capensibufo*. Node support is shown by posterior probabilities above each branch, and parsimony/maximum likelihood bootstrap values below; T, type localities.

blocks, which form a basal polytomy with *C. rosei* (Fig. 2). Net sequence divergences (p distances) between mountain blocks were 8–10% for ND2 and 4–5% for 16S (Fig. 2; Table 1). These values are similar to those normally found between species rather than within species (e.g. Vences *et al.*, 2005; Loader *et al.*, 2006; Blackburn, 2008).

Given the resulting topology (Fig. 2), three backbone constraints were created to examine various hypotheses of monophyly: (1) monophyly of *C. tradouwi* clades 1 and 2; (2) *C. tradouwi* clade 1 monophyletic with all *C. rosei* clades; and (3) *C. tradouwi* clade 2 monophyletic with *C. rosei* clades. In all cases support was low, as indicated by the percentage of trees that agreed with the constraint (Table 2), and did not lend support to these alternative scenarios.

BIOCLIMATIC MODELLING – CURRENT MODEL

The current *C. rosei* model was built on two major variables, July rainfall and ecoregions (Table 3). For *C. tradouwi*, ecoregions and soils lent most weight to the model, together with June rainfall (Table 3). However, for both species all three winter rainfall months (June, July, and August) were found to contribute most highly in jackknife tests, using both test and training gain. The minimum training presence used as a threshold value for *C. rosei* and *C. tradouwi* collection localities was found to be 0.46 and 0.12, respectively.

The current bioclimatic model for *C. rosei* (Fig. 3A) shows that the suitable climatic space is highly disjunct for this species. Within *C. rosei*, the largest area of unsuitability falls between the Hottentots Holland Mountains and the Cape Peninsula (an area known as the 'Cape Flats'), with a minimum distance of 40 km between mountain ranges. There are also several other substantial gaps, usually in valleys between mountain ranges.

The present-day bioclimatic model for *C. tradouwi* provides a similar picture of fragmented climatic space (Fig. 3B). The most striking feature is the overprediction (i.e. predicted overlap) into the range of *C. rosei*. This occurs even at high levels of suitability (up to 90%), and is robust to changes in the model parameters. The model also predicts that *C. tradouwi* should be found in the Piketberg, an isolated inselberg close to the west coast. Predictions of suitable climatic space in the south-eastern Cederberg coincide with existing records that lack sufficient precision to be used in bioclimatic modelling (i.e. Boycott, 2004).

BIOCLIMATIC MODELLING – SCENARIOS

Both scenarios, using only the three most important variables (mean winter rainfall, ecoregions, and soil),

Table 1. Net sequence divergences (p distances) for *Capensibufo tradouwi* and *Capensibufo rosei* by sampling with 16S (upper matrix) and ND2 (lower matrix)

Species	Site	Breekkrans	Turret Peak	Keeromsberg	Groot-vadersbosch	Pakhuis Pass	Hawekwas	Limietberg	Klein River Mtns	Akkedisberg	Landdroskop	Jonaskop	Muizenberg Mtn	du Toitsberg
		tradouwi	tradouwi	tradouwi	tradouwi	tradouwi	rosei	rosei	rosei	rosei	rosei	rosei	rosei	rosei
tradouwi	Breekkrans	NA/NA	NA	0.040	0.042	0.002	0.049	0.049	0.039	0.042	0.039	0.040	0.043	0.041
tradouwi	Turret Peak	0.088	NA/NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
tradouwi	Keeromsberg	0.084	0.104	0.002/0.001	0.010	0.044	0.049	0.049	0.047	0.046	0.040	0.042	0.045	0.034
tradouwi	Grootvadersbosch	0.108	0.106	0.057	0.003/0.000	0.044	0.050	0.050	0.048	0.044	0.039	0.040	0.044	0.031
tradouwi	Pakhuis Pass	0.007	0.092	0.091	0.110	0.001/0.000	0.053	0.053	0.042	0.043	0.043	0.044	0.047	0.045
rosei	Hawekwas	0.118	0.117	0.110	0.109	0.121	0.000/0.002	0.000	0.039	0.038	0.013	0.008	0.035	0.036
rosei	Limietberg	0.115	0.116	0.107	0.110	0.118	0.000	NA/0.006	0.039	0.038	0.013	0.008	0.035	0.036
rosei	Klein River Mtns	0.102	0.108	0.098	0.111	0.105	0.071	0.070	0.002/0.003	0.006	0.030	0.032	0.036	0.029
rosei	Akkedisberg	0.107	0.112	0.105	0.115	0.110	0.074	0.074	0.001	NA/0.000	0.028	0.030	0.038	0.028
rosei	Landdroskop	0.105	0.114	0.101	0.104	0.108	0.041	0.039	0.079	0.081	0.003/0.000	0.005	0.026	0.026
rosei	Jonaskop	0.107	0.117	0.099	0.101	0.111	0.036	0.035	0.079	0.080	0.018	0.003/0.002	0.027	0.028
rosei	Muizenberg Mtn	0.104	0.117	0.114	0.125	0.106	0.104	0.101	0.102	0.107	0.102	0.108	0.002/0.000	0.029
rosei	du Toitsberg	0.110	0.115	0.103	0.112	0.111	0.081	0.078	0.086	0.090	0.090	0.092	0.098	0.002/0.005

Values on the diagonal (italicized) reflect within-site p distances (16S/ND2). Values in bold show estimates for different sites within the same mountain chain. NA, not estimated for within-site divergences (only one sample at a site), or because of the non-amplification of the 16S fragment (Turret Peak).

show suitable palaeoclimatic space as being very similar to the present-day models (Table 3). For *C. tradouwi*, the palaeomodel over-predicts the area of occurrence in the south-westernmost area within the distribution of *C. rosei* (Fig. 4). In general, increasing winter rainfall to the maximum levels experienced prior to the LGM (plus 40 mm precipitation of mean winter rainfall), produces slightly larger suitable climatic space for both species (albeit spread over a much wider area for *C. rosei*), resulting in a less fragmented climatic space than in the current model. Regardless, suitable climatic space is confined to montane areas, and the overall range changes little. However, the predicted area of occurrence increases considerably for *C. tradouwi* (Fig. 4A, B; Table 3). Reducing rainfall to the minima experienced prior to the LGM (minus 15 mm precipitation of mean winter rainfall) results in greater fragmentation of climatic space, although no areas for which we have accurate locality details have less than the minimum training presence threshold (Fig. 4C, D).

Table 2. Percentage of Bayesian and maximum likelihood topologies agreeing with each constraint, in which hypotheses of monophyly (A–C) were examined

Hypothesis	% Bayesian	% ML
(A) monophyly of *Capensibufo tradouwi* clades 1 and 2	18	13
(B) *Capensibufo tradouwi* clade 1 monophyletic with *Capensibufo rosei*	17	6
(C) *Capensibufo tradouwi* clade 2 monophyletic with *Capensibufo rosei*	19	14

DISCUSSION

Phylogenetic analyses show that isolation in montane habitats has resulted in divergent clades of *Capensibufo*. Clades correspond with mountain ranges, but
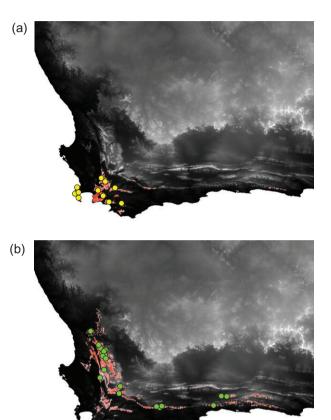
Figure 3. Bioclimatic model showing the suitability of areas above the minimum training presence for (A) *Capensibufo rosei* and (B) *Capensibufo tradouwi*. Darker shading indicates higher suitability.

Table 3. Percentage contribution of variables to the MAXENT model for *Capensibufo* using 51 variables, and percentage change in total extent and area of occurrence (EOO and AOO, respectively) under different palaeoclimatic regimes (mean winter rainfall maxima +40 mm and minima –15 mm) predicted to have occurred since the last glacial maximum

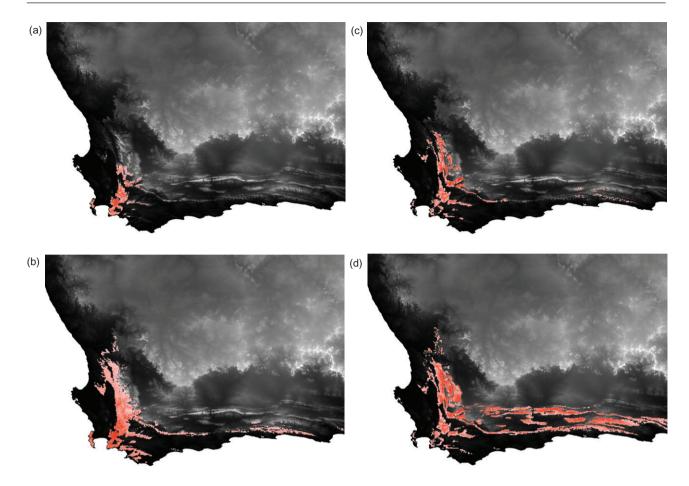| Variable name	*Capensibufo rosei*		*Capensibufo tradouwi*	
Ecoregions	33.9		51.3	
Soils	3.0		22.9	
Mean July rain	56.1		0.0	
Mean June rain	0.2		18.8	
Other variables	6.8		7.0	
	Min	Max	Min	Max
% change in EOO	–43.3	+251.0	–34	+7.2
% change in AOO	–41.0	+20.5	–36.0	+87.5

Figure 4. Bioclimatic model showing areas above the minimum training presence for *Capensibufo rosei* (A and B) and *Capensibufo tradouwi* (C and D) under different palaeoclimatic regimes representing the mean winter rainfall minima (−15 mm; A and C) and maxima (+40 mm; B and D) predicted to have occurred since the last glacial maximum. Darker shading indicates a higher suitability.

sites on the same mountain range (where sampled) are within the same clade, similar to the one mountain–one species model for *Arthroleptella* (e.g. Dawood & Channing, 2000; Turner & Channing, 2008). Sequence divergences between mountains are similar to those normally found between amphibian species, rather than within species (e.g. Vences *et al.*, 2005; Loader *et al.*, 2006; Blackburn, 2008). This suggests that montane isolation has produced species-level divergences, and that gene flow across mountain ranges in both species is extremely limited. This interpretation is consistent with bioclimatic models that show highly fragmented climatic space within the relatively large overall extent of occurrence. The bioclimatic models suggest that the climatic space of *Capensibufo* is defined mainly by ecoregion (vegetation type) and winter rainfall, which presumably corresponds to the availability of the seepages or ephemeral pools in which these toads breed. The distributions of these species may be limited prima-

rily by these factors, and suggest that suitable regions are extremely patchy and highly fragmented (Fig. 3). Furthermore, our results are consistent with predictions of low optimal range-expansion phenotype (ORP) probabilities for this genus, based on life-history traits including: small body size, absence of inguinal fat bodies, and small clutch size (Van Bocxlaer *et al.*, 2010).

Although these results call into doubt whether a two-species model is appropriate, there are some morphological differences that seem to correspond to a two-species model. *Capensibufo rosei* lacks a tympanum (but this is vaguely visible in a few large specimens) and an inner ear, and does not produce advertisement calls. Conversely, these characteristics are present in *C. tradouwi* (de Villiers, 2004b). These features are extremely important in maintaining (and promoting) species boundaries for amphibians (e.g. Menegon *et al.*, 2004). Furthermore, several other distinguishing morphological characteristics have been

noted (Grandison, 1980; du Preez & Carruthers, 2009), as have differences in egg production (Boycott, 2004): *C. rosei* lays strings of small eggs (2–3 mm), whereas *C. tradouwi* lays clutches of a few, individual, but larger eggs.

The apparent contrast between phylogenetic patterns and other lines of evidence presents an interesting dilemma when relying on genetic markers to validate species. Certainly, differences in morphology and life history/behaviour support the species status of these two species, although this is based on very little information overall (a handful of localities) on which to make good comparisons. Conversely, genetic markers point to divergences between mountain ranges (within so-called species) that are more reminiscent of the divergences normally observed between diagnosable species (e.g. *Arthroleptella*; Turner & Channing, 2008; see also Vences *et al.*, 2005). In this particular case, we suggest that confinement to isolated wet patches on mountain tops has promoted extraordinary 'within-species' genetic divergence, which is inconsistent with a two-species model. The main evidence for the two-species model relies heavily on the complete absence of advertisement calls in *C. rosei*. Despite this, there are very few call recordings of *C. tradouwi* (three from localities in the Cederberg, and one from Keeromsberg; H. Braak, A. Channing, M. Cunningham, A. Turner, pers. comm.), and these have never been analysed for differences (or similarities), making this entire line of evidence quite weak at present.

Grandison (1980) suggested that the wide Breede River valley forms a barrier between these two species. However, there are many other valleys that could also be considered to be strong barriers (see Fig. 1), and this is reflected in the resulting phylogeny. The question then arises as to the mechanism that has limited gene flow across these valleys. In fact, valleys have a very different set of environmental features ('climate') than mountain plateaus, and it may be that valley climates are unsuitable, resulting in climatic barriers. In addition, *Capensibufo* is dependent upon significant rainfall to form shallow breeding pools during winter, but the timing and intensity of this rainfall varies (see Schulze, 1997). *Capensibufo rosei* tadpoles have been never been found later than October (de Villiers, 2004b), concurrent with a decrease in rainfall at that time. Although based on only a few observations, it appears that *C. tradouwi* finishes breeding earlier, by September (Boycott, 2004), when rainfall diminishes within its distribution. These differences in timing are reflected in the month of winter rainfall that contributes the most to the bioclimatic model (Table 3). Indeed, the gap in distribution between these two species corresponds very well with the level of winter rainfall in

the August/September/October period (Fig. 5). Thus, gene flow via tadpoles or eggs washing into the watershed may be limited by the lack of rain during this most 'mobile' life stage. In fact, when rainfall is manipulated in the bioclimatic models (with both more and less rain), all the main gaps persist (Fig. 4), suggesting long-term isolation between individuals on mountain ranges.

Although there are several geographic divisions predicted by the model (and by the actual absence of the toads in these valleys), it should be pointed out that the climatic suitability of each species is over-predicted across the Breede River valley. The valley has very limited winter/spring rainfall (Schulze, 1997) during the time when adults would be migrating to/from breeding sites, and also later when tadpoles could be passively dispersed in streams (Fig. 5). This climatic feature could be an important factor contributing to maintaining the separation between the species, despite the climate being suitable on opposite sides of the valley. Furthermore, winter rainfall patterns have remained relatively stable during the Pleistocene (Chase & Meadows, 2007), possibly allowing for this separation to be maintained in the long term. However, because there is not yet a clear sense of whether there are many species, or two (genetically divergent) species, we cannot presume that the xeric conditions of this valley is the factor that separates the species in this genus.

CLIMATE MODELS AND GENETIC PATTERNS

The bioclimatic model shows each mountain block to be fairly distinct (or only partially connected), a condition that is reflected in the phylogeny. Sequence divergences for *Capensibufo* are much higher than that for other taxa from the region, suggesting that the radiation of these lineages is more ancient than the Plio-Pleistocene dates estimated for other Cape taxa (*cf.* Tolley *et al.*, 2006; Price, Barker & Villet, 2007; Smit, Robinson & Van Vuuren, 2007; Swart, Tolley & Matthee, 2009), and is probably on the order of middle or late Miocene. Mountain orogeny can be ruled out as promoting isolation, as the major uplift that formed the CFMs was predominantly in the Triassic, when the region was located over the south pole (McCarthy & Rubridge, 2005). Recent climatic shifts during the late Pleistocene (i.e. the limits of the bioclimatic model) can also be ruled out, as sequence divergences are much greater than would be expected over such a short time period.

Of particular interest is the Muizenberg Mountain sample site on the Cape Peninsula, which is isolated geographically and climatically (Figs 3, 4) from the other CFMs, and is notably divergent from the other *C. rosei* lineages (Fig. 2; Table 1). This mountainous
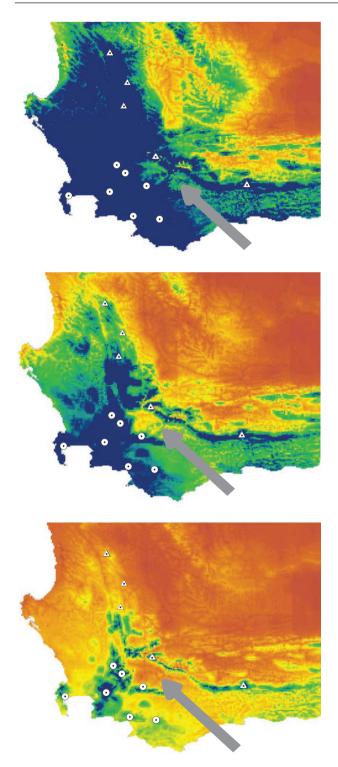
peninsula has probably sustained long-term isolation from the remainder of the CFMs because of repeated marine transgressions that have isolated the peninsula as islands (summarised in Mucina *et al.*, 2006), which is a pattern reminiscent of other Cape taxa. Southern rock agamas (*Agama atra*), western leopard toads (*Amietophrynus pantherinus*), and Cape platannas (*Xenopus gilli*) from the peninsula are particularly divergent from other CFM clades (Evans *et al.*, 1997; Swart *et al.*, 2009; Measey & Tolley, in press). However, estimated divergence times (Swart *et al.*, 2009; Measey & Tolley, in press) are not compatible with the high values for *Capensibufo* (e.g. 11% for ND2), again suggesting the processes affecting this taxon are dissimilar to, and pre-date, those for other Cape taxa. Interpretations regarding these processes, however, will require additional sampling sites, especially for *C. tradouwi*, and a dated phylogeny.

CONCLUSIONS

Overall, we conclude that *Capensibufo* conforms to the traditional amphibian model of low vagility, low gene flow, and strong genetic structure, and that these lineages have been separated on the order of millions of years. Given the size of the animal and the distances to be covered across inhospitable habitat, gene flow is probably a rare event. Because the bioclimatic models suggest that the distribution may remain fragmented, even with increased rainfall, the most likely scenario for species history is one of rare colonisation events into suitable habitat, rather than ever having had a 'continuous' distribution that is now fragmented. Generalist amphibians in the CFMs (e.g. *Strongylopus grayii*) may be able to overcome potential barriers to dispersal during rainy seasons by having prolonged breeding seasons and a broader breeding site preference. Indeed, *S. grayii* does not seem to be subject to similar restrictions as *Capensibufo*, nor does it show the strong structuring for the Cape peninsula, as do many other taxa (Tolley *et al.*, 2010). Thus, gene flow in amphibians should not be considered extremely unusual, and could occur across what seems to be inhospitable barriers, but the life-history constraints and low ORP for *Capensibufo* have resulted in extremely limited gene flow between mountains. Despite this, gene flow within a mountain range probably occurs, as *Capensibufo* sites within mountain ranges cluster together in terminal clades (e.g. Hawekwas). Other small amphibians in montane habitats are known to exhibit gene flow within a mountain range, especially where gene flow is mediated by watershed flow (e.g. Measey *et al.*, 2007a), and this could be the case for *Capensibufo*.

The taxonomic status of these species remains a matter of difficulty at this time. Each 'population'

Figure 5. Extent of winter rainfall in (A) August, (B) September, and (C) October within the distribution of *Capensibufo*. Warm/light colours indicate dry areas (dark red indicates driest), whereas cool/dark colours indicate wet areas (dark blue indicates wettest). The arrows point to the Breede River valley.

sampled is divergent at the species level, and calls into question whether there are in fact two species in this genus, or whether these lineages are divergent enough to be considered as separate species, perhaps conforming to the one mountain–one species model (see above). Additional work in the unsampled mountain ranges and greater attention to possible morphological, call, and ecological differences may eventually provide sufficient evidence to fully resolve this issue.

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