

# Investigating the cause of the disjunct distribution of *Amietophrynus pantherinus*, the Endangered South African western leopard toad

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Received: 16 April 2009 / Accepted: 3 September 2009 / Published online: 20 October 2009  
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**Abstract** More amphibians are threatened through loss of habitat than any other single factor. Conservation measures to restore habitat are dependent on historical data indicating the original extent of a species. When historical data is absent, disjunct distributions create a special problem for conservationists who need to determine whether they have an anthropogenic cause. The Endangered western leopard toad (*Amietophrynus pantherinus*) has a disjunct distribution in the south-western tip of South Africa. We use mitochondrial sequences from 153 individuals to show that the disjunct distribution is unlikely to have an anthropogenic origin. Two distinct populations are separated by 100 km, with highest probability for the most recent common ancestor arising some 5 Kya. The causes of this disjunct distribution appear to be too recent to be attributable to changes in sea level; instead we suggest that there was a range retraction associated with a distinct drying period in the area during the Holocene, possibly indicating that this species will be susceptible to future climate change. Further, we find that the eastern population is less genetically diverse and appears to be undergoing a serious reduction in range, despite its occurrence in the least urbanized habitat. Conservation measures suggested

include surveys for breeding sites in the eastern population, custodian agreements for existing breeding sites and foraging areas and the need for a Biodiversity Management Plan to be drawn up and implemented.

**Keywords** Cape floristic region · Amphibian decline · Landscape genetics · mtDNA · Bufonidae

## Introduction

Concern over amphibian declines has produced a plethora of research into the reasons for so-called ‘enigmatic declines’, providing evidence for a diverse array of causes from a sometimes benign, sometimes lethal fungus (*Batrachochytrium dendrobatidis*) to climate change (Daszak et al. 2003; Stuart et al. 2004; Pounds et al. 2006). However, the proximate cause of global biodiversity loss is through habitat change (Millennium Ecosystem Assessment 2005) which ultimately stems from increasing human population and per capita consumption (Pletscher and Schwartz 2000). While agriculture affects by far the largest areas, the most severe and destructive of land use change is through urbanization. Indeed, the greatest loss to amphibian species diversity across a North American landscape was through urbanization (Knutson et al. 1999). The types of potential barriers to migration and dispersal of amphibians in an urban environment is very large, but the most common are roads, canalized or piped waterways and walls. Concerns over amphibian decline have also seen resurgence for studies focussing on the plight of amphibians in urban environments. Their methods include surveys, population genetics, geographical landscape analysis and radio-tracking individual animals (Pellet et al. 2004; Rubbo

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and Kiesecker 2005; Husté et al. 2006; Parris 2006; Gagné and Fahrig 2007; Noël et al. 2007), and most focus on fragmentation and identifying barriers to movement.

Pond-breeding amphibians, which make up the majority of amphibian species (Duellman and Trueb 1986), are biphasic, making use of two habitat types and often undergoing yearly migrations between them (Semlitsch 2008). Traditionally, it is considered that there are two major types of movement: migrations, by adults from foraging to breeding sites at the metapopulation level (cf. Marsh and Trenham 2000), and dispersal, by sub-adults, between populations (Semlitsch 2008). Studies showing unbiased dispersal of amphibians exist (Smith and Green 2006; Cabe et al. 2007), and the metapopulation interpretation of amphibian population structure has been questioned (e.g. Jehle et al. 2005). In addition, pond breeding amphibians are often considered to show breeding site fidelity, but a growing number of genetic studies are revealing movements by a sub-set of individuals between neighbouring ponds (e.g. Newman and Squire 2001; Jehle et al. 2005; Arens et al. 2007). However, the general principle of two types of movement (migration and dispersal) at different life-history stages (adult and sub-adult, respectively) may hold for the majority of individuals in the majority of pond-breeding species (Semlitsch 2008).

Amphibians are typically described as poor dispersers (e.g. Blaustein et al. 1994), and their populations in most cases show a strong phylogeographic structuring (Avice 2000). However, evidence for long distance dispersal in ecological studies suggest that at least some species are far from sedentary; instead regular dispersal by a few individuals may cover several kilometres (see Smith and Green 2005 for a recent review). For example, one mark-recapture study of North American toads showed around 2% of toads moving more than 2 km, while one adult individual was found to have moved 34 km (Smith and Green 2006). In a different approach, Hu et al. (2007) explored the genetic structure of a widespread Chinese toad, finding that long-distance dispersal and colonisation, not vicariance, is responsible for present day distribution of haplotypes. It could be predicted that dispersal might vary across families of amphibians, but it already appears that large toads are capable of quickly dispersing over many kilometres (e.g. Phillips et al. 2006).

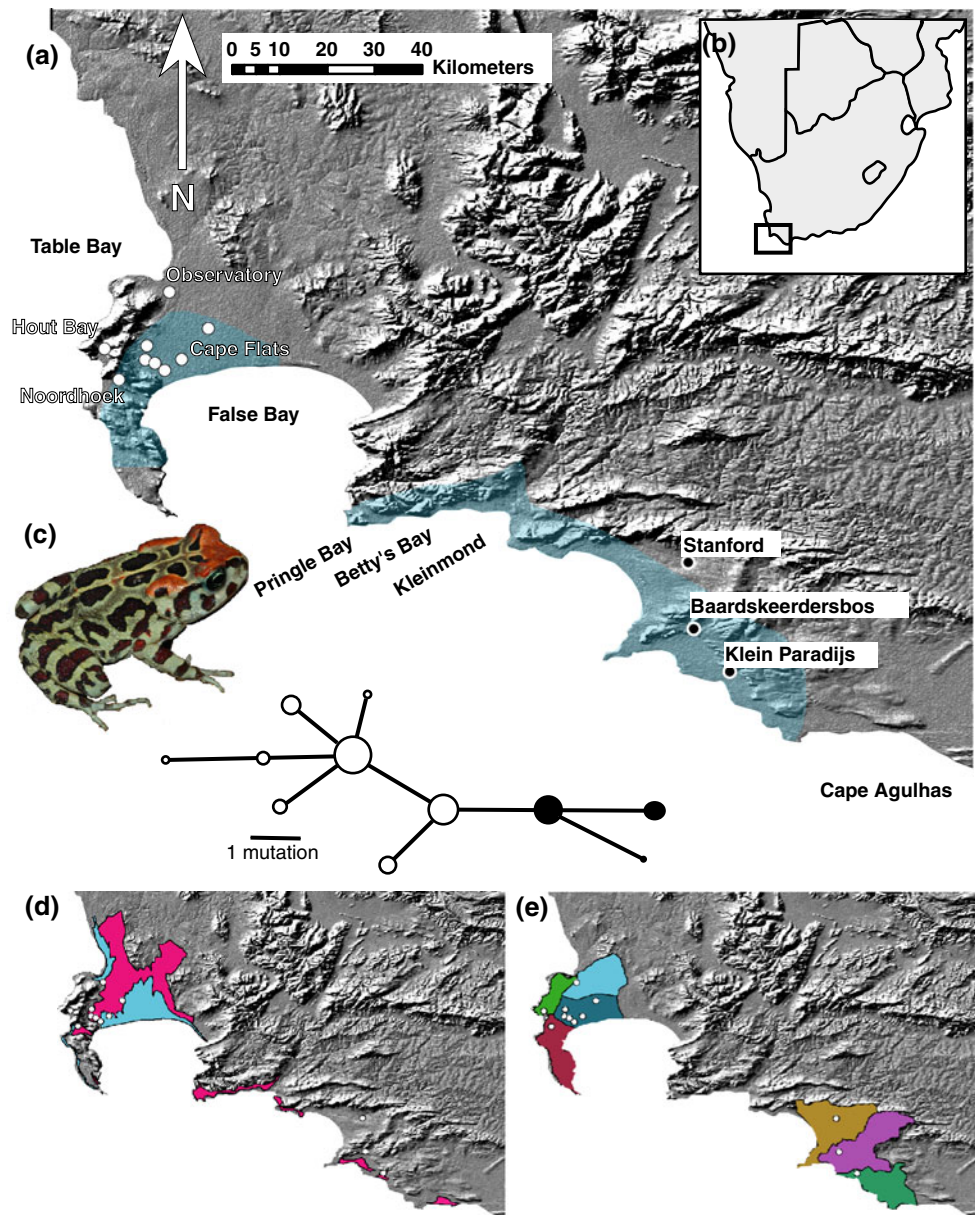
The Cape Floristic Region, of which the Cape Metropolitan area is a part (see Fig. 1a) has long been recognised as a global biodiversity hotspot and is a conservation priority (Myers et al. 2000). The area has a particularly high topographic heterogeneity, supporting a high number of habitats and ecological communities (Cowling et al. 1996). The peninsula area is exceptionally rich in plants with some 2,285 species, of which 90 are endemic and 141 threatened (Trinder-Smith et al. 1996). While human

occupation of the Cape Peninsula dates back at least 200,000 years BP, it is unlikely that large scale habitat change occurred until after colonial settlement in 1652 (Cowling et al. 1996), and then only on a large scale within the past 200 years. The metropolitan area of Cape Town and surroundings is home to some 3.2 million human inhabitants (an official but probably conservative estimate), as well as eight threatened amphibian species (IUCN et al. 2006) of which only two (*Heleophryne rosei* and *Capensibufo rosei*) do not occur on the highly urbanized “Cape Flats” (Minter et al. 2004). Five of these threatened amphibians (*Amietophrynus pantherinus*, *Capensibufo rosei*, *Hyperolius horstockii*, *Microbatrachella capensis* and *Xenopus gilli*) have a distribution across the Cape Flats mainly on Sand Fynbos and, with a gap roughly corresponding with the intrusion of Strandveld Fynbos; a heathland endemic to the South African cape region (Fig. 1d; see Mucina et al. 2006).

For conservationists, species with disjunct distributions present a quandary over whether connectivity needs to be restored or discrete populations should be separate management units. This can be exasperated when historical data is absent or ambivalent, such as museum specimens which have “South Africa” or even “Africa” as their locality (e.g. Poynton and Lambiris 1998). Genetic studies can be used to infer historical states through a number of analytical techniques. For example, a molecular study of the Cape Platanna (*Xenopus gilli*), showed a genetic division across the Cape Flats gap (Evans et al. 1997). Recognition of genetic structure in the Cape Platanna was notable, as it led to separate conservation management plans for these two lineages (de Villiers 2004b). This disjunct pattern extends to numerous other species that might encounter migrational difficulties across a saline intrusion due to higher sea levels, including the proteas *Leucospermum hypophyllocarpodendron hypophyllocarpodendron* and *Mimetes hirtus*, the fresh water amphipod *Mesamphisopus capensis*, and *Agama atra*, the southern rock agama (Rourke 1972, 1984; Gouws et al. 2004; Swart et al. 2009).

The western leopard toad (*A. pantherinus*) carries the Endangered status [EN: B1ab(ii, iii, iv, v) + 2ab(ii, iii, iv, v)] due to its small distribution within the winter rainfall region of the Western Cape, and the ongoing reduction in quantity and quality of habitat associated with urbanisation (de Villiers 2004a). Within the Cape Metropolitan area, the sites this species inhabits are almost completely urbanized; with canalized rivers and channels, major roads and scant foraging areas packed with residential dwellings. In contrast, east of the Cape Flats the habitat is far less disturbed, although even this area has undergone considerable urbanisation and agricultural expansion in the last 20 years. Aside from some behavioural and taxonomic studies

**Fig. 1** **a** Study area from the Peninsula to Agulhas in the Cape region of South Africa. **b** Shaded area shows the disjunct distribution of the western leopard toad (*Amietophrynus pantherinus*) on the Cape Metropolitan area (CMA, westernmost) and East of False Bay (EFB), from the most recent red-list assessment (Minter and Harrison 2004). White filled circles are sampling sites from this study in the CMA, while black filled circles are sampling sites EFB. Note that three sampling sites are outside of the previously known distribution, but that no toads were found in Pringle Bay, Betty’s Bay or Kleinmond. **c** A median-joining network of haplotypes (ND2 mitochondrial gene) for *A. pantherinus*. Haplotypes are represented by circles and show the proportion of toads from CMA (white) and EFB (black). The area of the circle is proportional to the number of individuals with that haplotype, and the length of the connecting lines is proportional to the number of base changes between haplotypes. **d** Distribution of two vegetation types within the study area: Strandveld Fynbos (dark shading) and Sand Fynbos (light shading; (Mucina and Rutherford 2006). **e** Quaternary catchments of all areas with sampling sites



(Passmore 1977; Cherry 1992a, b; Poynton and Lambiris 1998; Eick et al. 2001), very little is known about the population biology of this species, especially in a context that might help with conservation planning.

In this study, we set out to investigate whether the disjunct distribution of *A. pantherinus* across the Cape Flats is an ancient division (as suggested for *X. gilli*) or a result of modern habitat loss (i.e. within the past 200 years). Secondly, we aimed to determine whether the currently fragmented and highly disturbed breeding sites within the Cape Metropolitan area represent a single population with little or no genetic structure, or whether there are indications of structure associated with breeding areas.

**Materials and methods**

**Study species**

The western leopard toad (*Amietophrynus pantherinus*) is a large bufonid; typical size for adult males and females is between 90 and 110 mm (Cherry 1992b). After its description in 1828 (as *Bufo pantherinus*), this species spent a long period in synonymy, and was known under a variety of different names until the designation of a neotype by Poynton and Lambiris (1998). Since then, preliminary molecular studies comparing it with *A. pardalis* suggest that *A. pantherinus* is a distinct lineage (Eick et al. 2001;



Cunningham and Cherry 2004), although a comprehensive assessment of the same is still needed. Metamorphosed individuals have striking dorsal patterning of chocolate-brown patches surrounded by bright yellow on a light brown background (see Fig. 1). The distribution of *A. pantherinus* is within the winter rainfall zone of Western Cape Province, South Africa where it falls into two disjunct areas: east and west of False Bay and the Cape Flats (Fig. 1a; Minter and Harrison 2004). Adults are explosive breeders, moving to breeding sites during the antipodean winter (late July to early September) where they lay large clutches of spawn in strings (Cherry 1992b; de Villiers 2004a). Tadpoles take around 3 months to reach metamorphosis, and males are known to breed after 1 year and females after two (Cherry 1992a). During migration and dispersal, many individuals are killed on roads adjoining breeding sites (de Villiers 2004a).

### Sampling

We conducted sampling for tissues during the breeding season in August 2007, with subsequent follow up sampling of tadpole tail tips from certain areas in September and October. Tissue samples were always taken from road casualties, where these were available, and we supplemented these by taking a single phalange from the outside toe of the left foot from live adults. Toe-clipping is an established procedure for tissue sampling of live adult anurans and despite some controversy this method is known to have minimal effect on terrestrial species when <5 digits are removed (Funk et al. 2005). We attempted to sample individuals from each drainage catchment within the known distribution (Fig. 1a, e).

### DNA isolation and sequencing

When considering appropriate genetic markers for population level analysis we followed Cunningham and Cherry (2004) in their study of 20-chromosome toads by choosing the ND2 marker which is thought to have sufficient variation at the population level. Total genomic DNA was isolated from tissue samples stored in 98% ethanol using a standard salt extraction (Bruford et al. 1992). We amplified ND2 from vMet2 (GCT AAA CAA GCT TTC GGG CCC ATA CC) to vTrp (CTC CTG CTT AGG GCT TTG AAG GC) situated in the highly conserved methionine and tryptophan tRNA genes flanking ND2 (Cunningham and Cherry 2004). PCR annealing temperature was optimised at 57°C (40 cycles) and magnesium concentration was 2.5 mM MgCl<sub>2</sub>. Amplified PCR products were sent to Macrogen Inc., Korea for sequencing.

### Data analysis

A spatial analysis of variance (SAMOVA) was conducted on the entire data set to determine *a priori* clustering of sampling sites (Dupanloup et al. 2002). This analysis used the ND2 haplotype data and geographic co-ordinates of each individual toad sampled to statistically differentiate between groups of sample sites that presumably represent populations. The SAMOVA was run for  $K = 2-5$  groups to determine the maximum value of  $F_{CT}$ , the maximized proportion of total genetic variance due to differences between groups (Dupanloup et al. 2002). Subsequently, haplotype ( $h$ ) and nucleotide ( $\pi$ ) diversity were estimated in ARLEQUIN (v2.011 Schneider et al. 2000) for each population predetermined by SAMOVA. In addition, relationships among haplotypes were examined with a median-joining network in Network 4.1 (Bandelt et al. 1999).

A coalescent approach using the software IMA (Hey and Nielsen 2007) was used to investigate the populations defined by SAMOVA. We explored the relative size of populations ( $\theta_A$  for ancestral population size,  $\theta_1$  for population 1 and  $\theta_2$  for population 2), migration rate for both populations ( $m$ ), divergence time ( $t$ ) and estimated time of the most recent common ancestor of the two populations (TMRCA). Marginal posterior probability densities with 95% upper and lower limits for these parameters were recorded. The IMA software uses a Felsenstein framework to run Markov chain Monte Carlo (MCMC) simulations permitting likelihood-based analyses (Hey and Nielsen 2007). Data were submitted to the remote computer cluster running the program IMA at Cornell University via internet upload (<http://cbsuapps.tc.cornell.edu/IMA.aspx>). Tuning of the priors was performed manually following preliminary analysis runs and guided by the output reports. Initial runs estimated migration as zero, so re-runs were performed with the priors for  $m_1$  and  $m_2$  set to zero. Three independent runs of IMA were carried out to ensure repeated convergence on the same results. Each run utilised 100 Markov chains, two million burn-in steps, 10,000 steps of the MCMC simulation (sampling every 1000 generations). A geometric heating scheme was used with  $g_1 = 0.9$  and  $g_2 = 0.8$ , and 100 chain swap attempts per Metropolis-coupling step. We used a rough estimate of a point mutation rate of 0.69% per million years for ND2 (Macey et al. 1998), and a generation time of 2 years (Cherry 1992a) to estimate time in years to TMRCA. We acknowledge the estimate could be biased due to the use of a single, fixed mutation rate which lacks estimates of confidence.

Isolation by distance among breeding areas in the Cape Metropolitan area (CMA) was examined whereby toads were geographically assigned to the breeding site closest to their capture. Amphibians are generally considered to be

philopatric (Avice 2000; Semlitsch 2008), and studies in continuous habitat have typically shown isolation by distance patterns (Cabe et al. 2007). A Mantel test with 10,000 permutations was used to compare the pairwise matrix of  $F_{ST}$  values (as a proxy for genetic difference) with the Euclidean distances between breeding areas, using Mantel for Windows version 1.18 (Calvacanti 2005).

In addition to the SAMOVA, an a posteriori analysis of molecular variance (AMOVA Excoffier et al. 1992) was run in ARLEQUIN on individuals within the CMA to estimate levels of variation under three different hypotheses of population differentiation to investigate landscape influence on genetic structure (cf. Manel et al. 2003). First, we hypothesised that sets of breeding sites within 1 km of each other (i.e. breeding areas) would constitute populations, following Semelitsch’s (2008) report that 99% of toads are found within 850 m of their breeding site. Second, we hypothesised that populations would be defined by mountain barriers. Essentially this grouped sample sites within the CMA into three areas: Cape Flats, Hout Bay and Noordhoek (Fig. 1a). Third, we hypothesised that quaternary catchment areas would define populations. Four catchments in the Cape Metropolitan area corresponded to sampling sites in Observatory, Hout Bay, Noordhoek and southern Cape Flats sites (Fig. 1a, e).

**Results**

We obtained samples ( $n = 110$ ) from nine sample sites representing all four of the known catchment areas in the Cape Metropolitan area (CMA), and three sites East of False Bay (EFB;  $n = 43$ ; Table 1). Despite searches from Pringle Bay to Kleinmond (Fig. 1a), no western leopard toad breeding sites were found and no individuals were sighted. Within the CMA, we obtained samples from several previously unknown breeding sites at newly constructed artificial ponds in Noordhoek, Hout Bay and Tokai (Fig. 1a). Individuals sampled from Observatory were not from a known breeding site, although it is presumed (due to a number of metamorphs found there) that a breeding site exists within the immediate area of where individuals were found.

**Examination of distribution gap**

Sequences from an 805 base pair fragment of ND2 were obtained from a total of 153 individual western leopard toads which were represented by 11 haplotypes. The SAMOVA analysis revealed an unambiguous interpretation for two populations with the maximum value of  $F_{CT}$  (0.6164), which sharply dropped off for with larger values of  $K$ . These two populations conclusively corresponded to

**Table 1** Haplotype frequency and distribution for the western leopard toad, *Amietophrynus pantherinus*, in the Cape metropolitan area (CMA) and East of False Bay (EFB), together with their genbank accession numbers

Haplotype	Frequency	CMA	EFB	Genbank
HAP_1	13	0	13	GQ994942
HAP_2	1	0	1	GQ994943
HAP_3	46	0	46	GQ994944
HAP_4	6	0	6	GQ994945
HAP_5	4	0	4	GQ994946
HAP_7	31	0	31	GQ994947
HAP_8	27	27	0	GQ994948
HAP_9	15	15	0	GQ994949
HAP_10	8	0	8	GQ994950
HAP_12	1	0	1	GQ994952
HAP_13	1	1	0	GQ994953
Total	153	43	110	

sites sampled EFB and those in the CMA. Haplotype ( $h$ ) and nucleotide ( $\pi$ ) diversity were greater for the population in the CMA than EFB as indicated by the 95% CIs (Table 2). This pattern is reflected in the haplotype network (Fig. 1c) in which no haplotypes were shared across the two populations and the number of haplotypes were greater in the CMA.

**Coalescent analysis**

Results from IMA show relatively large differences in population size, with the CMA population around five times larger than the EFB population (Table 3). The common ancestral population was estimated to be around the same size as that currently found EFB, although estimates of confidence around this are large (Table 3). The divergence time ( $t$ ) is estimated as 1.2 thousand years ago (confidence estimates 0.4–3.4 Kya) and time to the most recent common ancestor (TMRCA) is estimated as 4.75 thousand years ago (confidence estimates 2–55 Kya; Table 3).

**Structure within the cape metropolitan area**

Results from the Mantel test for isolation by distance show no overall association between  $F_{ST}$  and geographical distance ( $r = -0.235$ ;  $P = ns$ ), which suggests that gene flow does not follow a simple isolation by distance pattern within the CMA. However, a posteriori AMOVA tests did show significant genetic structuring for two of the scenarios investigated (grouped by breeding area, grouped by mountain barrier), but not by catchment (Table 4). The  $\Phi_{ST}$

**Table 2** Number of individuals ( $N$ ), number of haplotypes, and molecular diversity measures ( $h$  and  $\pi$ ) for populations Cape metropolitan area and East of False Bay (see Fig. 1a)

Site group	$N$	Haplotypes	Gene diversity ( $h$ )	Nucleotide diversity ( $\pi$ )
East of False Bay (EFB)	43	3	0.4950 $\pm$ 0.0489	0.000636 $\pm$ 0.00061
Cape metropolitan area (CMA)	110	8	0.7286 $\pm$ 0.0278	0.001287 $\pm$ 0.00096

**Table 3** Mean results of three runs of IMA on populations of Western leopard toads from the Cape metropolitan area (CMA) and East of False Bay (EFB)

	Parameter estimate	Lower	Upper
$\theta$ CMA	6.26	2.92	14.52
$\theta$ EFB	1.82	0.59	8.88
$\theta$ A	0.35	0.24	15.37
$t$ (Kya)	1.19	0.40	3.45
TMRCAs (Kya)	4.75	2.18	56.85

Parameter estimates are the most probable values from posterior density curves, whereas the lower and upper limits are the values around which 95% of the posterior density function falls; estimates were made for the relative population size ( $\theta$ ) for each population and for the ancestral population ( $\theta$  A), divergence time ( $t$ ) and the time to the most recent common ancestor (TMRCAs), see Fig. 2

values were much higher for breeding area groupings than for mountain barrier groupings.

## Discussion

Population analysis of sequence data generated in this study suggests that *Amietophrynus pantherinus* is geographically and genetically partitioned by a distribution gap across the Cape Flats. Results from the coalescent analysis suggest that gene flow across this gap may have ceased as recently as 1.2 thousand years ago, and that gene flow at present is absent. Thus, the gap in the distribution cannot be ascribed to modern human habitat change within the last 200 years. Further, the most recent common ancestor for both populations existed some 4.75 thousand

years ago. Toads sampled from breeding sites within the Cape Metropolitan area (CMA) do not show a pattern of isolation by distance, but instead form groups associated with breeding areas.

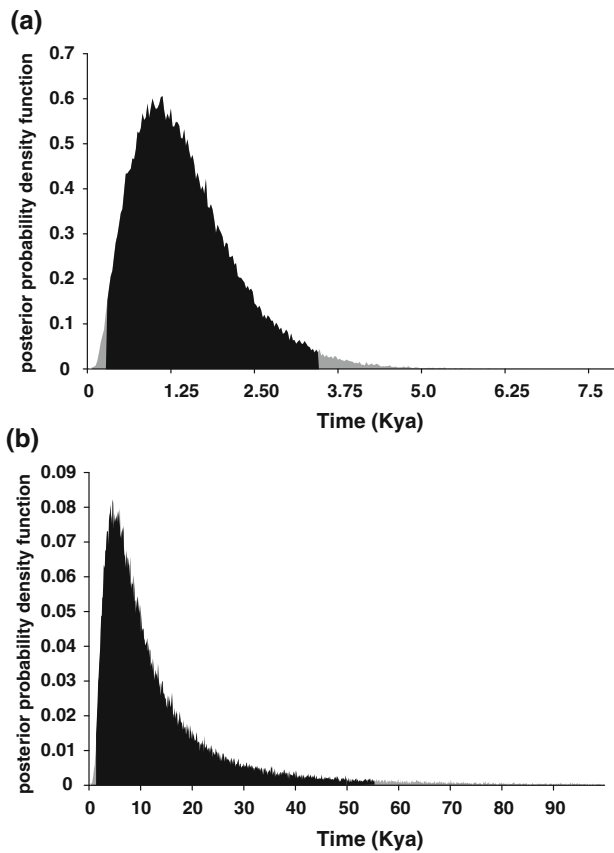
### Causes of disjunct distribution

In their discussion of the disjunct distribution of *Xenopus gilli*, Evans et al. (1997) suggested that gene flow was interrupted by oceanic transgression of the Cape Flats. Certainly the ocean is thought to have covered the Cape Flats during the early Pliocene (3–4 Ma) when sea levels were thought to be up to 35 m higher (Wardlaw and Quinn 1991). However, oceanic regressions, rather than transgressions, may actually be more important. During the last glacial maximum sea levels were around 125 m lower (Lambeck 2004), exposing the shallow Agulhas Bank and forcing the seashore more than 140 km southwards (van Andel 1989). The resulting habitat may have provided many paths for gene flow of terrestrial fauna and flora which later became disjunct (cf. Swartz et al. 2007). The present day situation represents an intermediary between a maxima which last flooded the Cape Flats and turned the Cape Peninsula into an archipelago of islands 1.5 Ma, and a minima which exposed the entire Agulhas Bank leaving a much larger terrestrial area as recently as 17 ka (van Andel 1989; Lambeck 2004).

For western leopard toads, our data suggest a cessation of gene flow only 1.2 thousand years ago. Data on sea level changes within the last 8,000 years suggest that fluctuations were all within 4 m of present sea levels, (Baxter and Meadows 1999) having little effect on the position of the

**Table 4** Results from analysis of molecular variance (AMOVA) with eight sample sites grouped by (1) breeding areas, (2) mountain barriers, and (3) catchments (see text)

Groups	No. of groups	Variance components	% variation $\Phi_{ST}$	$P$ -value
1. Separated by breeding area	8	Among groups	18.23	<0.05
		Among sites	–2.45	
		Within sites	84.22	
2. Separated by mountains	3	Among groups	0.84	<0.05
		Among sites	14.98	
		Within sites	84.18	
3. Separated by catchments	4	Among groups	–1.58	NS
		Among sites	16.87	
		Within sites	84.71	



**Fig. 2** Three independent replicates of the posterior probability density functions for **a** the divergence time and **b** the most recent common ancestor of western leopard toads. *Upper* and *lower* limits around which 95% of the posterior density function falls are *shaded*

coastline. Thus, our results cannot easily be explained by oceanic transgressions, or regressions. An alternative explanation for significant changes within the Holocene period may be the considerable drying of the climate in the region (Chase and Meadows 2007). Two scenarios (which are not mutually exclusive) for the past distribution of this species are plausible given our findings. First, that the distribution of the western leopard toad was much larger (i.e. including both sides of False Bay, and possibly further) and was subsequently fragmented with the drying climate. Second, that the past distribution was limited to the Cape Peninsula area (and presumably the increased land area that existed to the south, see above), with individuals colonising areas East of False Bay during favourable conditions which ceased with increasing aridification 1.2 thousand years ago. This second scenario might fit better with the distribution of haplotypes, which have been interpreted in other studies on toads to be the result of long-distance migration (Hu et al. 2007). These scenarios might be tested using niche models based on current climate data and run with past climate scenarios to elucidate the probable past distribution of this and other Cape amphibians.

Increased temperatures and a reduction in precipitation might be expected to influence toad dispersal. Western leopard toads presently occur within Sand Fynbos, which is acidic, while the geographic gap corresponds with Strandveld Fynbos which is alkaline (Harrison 1962). During a drying climate, pH of water in potential breeding sites would become more strongly influenced by the substrate. Low pH is known to be necessary for successful reproduction of the Cape Platanna (Picker 1993), and while data is lacking regarding preferences for *A. pantherinus*, suitable experiments could be easily conducted.

As our estimate for the timing of the disjunction falls during the Holocene, the consequences of a changing climate during this period (Chase and Meadows 2007) is the most likely causal agent of division between the populations. It would be worthwhile to conduct similar studies to date separations in other Cape fauna and flora with similar disjunct distributions to discover whether these were caused by recent climatic fluctuations or more ancient oceanic transgressions. Importantly, species which have already been impacted by recent (Holocene) climate change may be considered to be more vulnerable to ongoing climatic fluctuations.

Our results are not consistent with a continuous population of western leopard toads across the Cape Flats within the past 200 years. Despite the large confidence estimates and the use of a single fixed mutation rate, the lower estimate for divergence time was around 400 years BP, with the most likely estimate approximately 1200 years ago. We therefore consider that it is unlikely that recent anthropogenically mediated habitat change on the Cape Flats is responsible for the current disjunct distribution of this species.

Relative rates of migration from the coalescent analysis are estimated to be zero between populations in CMA and EFB. A few individuals of some toad species have made remarkably large movements during migration and dispersal (e.g. Sztatecsny and Schabetsberger 2005; Smith and Green 2006), but no toad has been recorded to move 100 km (the smallest possible distance between known breeding sites east and west of False Bay; see Fig. 1a). This is consistent with the separation of populations by SAM-OVA, and it may be presumed that this is the result of ancestral panmixia or long distance migration across False Bay.

#### Genetic differentiation within CMA

For amphibians, determining the genetic structure is important to support conservation decisions because the information may be used to (1) determine connectivity of breeding sites, and (2) provide information on areas suitable for rehabilitation of both breeding sites and foraging

areas. This would obviously lead to improved management of amphibians under threat. For the threatened western leopard toad, our results suggest that there is not a simple pattern of isolation by distance within the CMA as in some amphibians from other regions (Cabe et al. 2007). Instead, we find that breeding areas contain distinct assemblages of haplotypes. Toads have traditionally been regarded as being philopatric to breeding ponds, but some fine-scale genetic studies have revealed significant gene-flow between ponds (Shaffer et al. 2000; Martinez-Solano and Gonzalez 2008). As adult western leopard toads colonise new breeding sites (ponds and slow flowing channels) relatively quickly (within 2 years of their construction; G.J. Measey, personal observation), significant gene-flow might be expected within migration distances (Semlitsch 2008).

While preferring still water, western leopard toads regularly deposit eggs in slow flowing streams during antipodean winter when they are vulnerable to passive dispersal by swelling of streams and rivers due to winter rains (G.J. Measey, personal observations). Movement of tadpoles and (to a lesser extent) eggs in flowing water may result in structuring over breeding areas instead of breeding sites. This factor may therefore be more important for dispersal of genes than any movement of adults. Determining the nature of this gene-flow for this species is regarded as important in management of western leopard toads. While there is strong philopatry of these toads to their breeding areas, more studies using variable microsatellite markers, may discover whether individual ponds or breeding areas represent meta-populations (Marsh and Trenham 2000).

### Conservation

Prior to the 1990s, breeding sites were known from three other (herewith unsampled) localities (Pringle Bay, Betty's Bay and Kleinmond; Fig. 1a) in the EFB region (see Minter et al. 2004), and we hypothesise that these sites also belonged to the EFB population. Unfortunately, the lack of any sightings in recent years has made that hypothesis untestable. Although we cannot discount that breeding sites still exist, breeding has not been reported in those areas for more than 25 years (de Villiers 2004a), and the last lone individual seen in this area was prior to 1995 (A. de Villiers, personnel communication). It appears that toads have become locally extinct in these areas. Our study provides additional evidence that toads in the EFB region may be of greater conservation concern than previously realised. Their genetic diversity is lower than those of the CMA, and the coalescent analysis estimates of population size were notably smaller than the CMA population despite distributions covering similar sized areas (Fig. 1). However, we acknowledge that this study with a single mtDNA marker may not be adequate to reflect true genetic diversity given

the apparently recent disjunction from populations in the CMA (see above).

Despite these caveats, relatively low genetic diversity coupled with apparent local extinctions and a small number of remaining breeding sites may be warning signs that the EFB population is in grave danger. While this area has not been prioritised for conservation effort (de Villiers 2004a), this population appears to be more vulnerable than the CMA population, despite the area being less heavily urbanised. Although the cause may be historical (see above), the problem is certainly exacerbated by ongoing development in the EFB lowlands as much of the remaining lowlands are being utilised for agriculture and tourism. This trend is likely to increase as the area is considered to have a low habitat fragmentation index, high crop potential, and medium potential for population density increase, despite the biome having a low resilience to habitat change (Driver et al. 2005). We suggest that the results of this study show that the population EFB needs to be treated as a separate management unit, and that some urgent action is taken to ascertain whether historical breeding sites in Pringle Bay, Betty's Bay and Kleinmond are still viable, and to safeguard the future of those breeding sites remaining. The CMA population is presently receiving conservation attention, due to the perceived threats such as road casualties, invasive fish (predation on tadpoles) and general urbanisation leading to fragmentation and habitat loss. Ironically, it may be that the more rural EFB population is in greater danger.

### Future conservation actions

Despite its relatively small extent of occurrence, the western leopard toad forages and breeds in sites owed by numerous stakeholders, the majority of which are members of the public living in residential areas within the CMA. This species also faces a large number of threats which operate from the individual to population level throughout its range (de Villiers 2004a). South Africa has recently implemented new legislation for managing threatened species: Biodiversity Management Plans (BMP in National Environmental Management: Biodiversity Act, 2004, Act no. 10), and a BMP for the western leopard toad is currently being written by representatives from all stakeholders who meet regularly as the Western Leopard Toad Conservation Committee.

In this study, we have highlighted the urgent need to reassess the presence of this species in previously known sites in the EFB, increase the resolution of genetic studies in the CMA, and finally, the need for a BMP. The status of known breeding sites (all of which occur on private land) and foraging areas in the EFB should be consolidated, preferably with the use of custodial agreements. Within the



CMA, we suggest that mark-recapture and radio-tracking of individual toads after breeding will allow for a better understanding of their use of urbanised spaces during migrations. In turn, this will allow more timely management by the City of Cape Town council of recreational areas where these abut breeding sites, as well as management of drainage which does not coincide with breeding activities. Radio-tracking and mark-recapture of adults would augment fine scale genetic work (using microsatellites) to provide more information concerning dispersal of this threatened amphibian, and such information will be extremely valuable for understanding population structure.

**Acknowledgments** This project received funding from SANBI Applied Biodiversity Research, Molecular Ecology group. Field work was carried out under a CapeNature permit (#AAA004-00090-0035) and received an ethical clearance certificate (# 0001/08 from SANBI Ethics Committee). We are grateful to the many volunteers and professionals who have passed on toad carcasses and tissue samples used in this study, especially: Maya Beukes, Atherton de Villiers, Mark Day, Cliff and Suretha Dorse, Farrah Feldman, Grant Forbes, Dalton Gibbs, Sandra Hollerman, Kareemah Jacobs, Suzie Jirachareonkul and Evanne Rothwell. Thanks also go to Brian Chase, Michael Cunningham, Atherton de Villiers, Tony Rebelo and Andrew Turner for helpful discussions. An anonymous reviewer gave insightful comments on an earlier draft manuscript. SANBI field and lab work was greatly helped by Lucas Chauke, Keshni Gopal, Kevin Hopkins and Jenny Underhill.

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