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The Conservation Genetics of a Newly Recognised Cape Peninsula Endemic: Rose's Mountain Toad (*Capensibufo rosei*)

EMILY R. CRESSEY[1]

Supervisors: Dr Krystal Tolley[2], Dr John Measey[2],

and Associate Professor Peter Ryan[1]

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University of Cape Town

[1]*Percy FitzPatrick Institute, University of Cape Town, Rondebosch, 7701, South Africa*

[2]*Applied Biodiversity Research Division, Kirstenbosch Research Centre, South African National Biodiversity Institute, Private Bag X7, Claremont 7799, South Africa*

email: em cressey@yahoo.co.uk

ABSTRACT

Declines and losses of amphibian populations are a global problem involving a complexity of interacting causes. Regardless of the fact that amphibians in Africa are among those predicted to be hit the hardest by anthropogenic global change, many species remain poorly studied. *Capensibufo rosei*, Rose's Mountain Toad, is a restricted range species that survives in a few small, isolated montane populations in the extreme south-western Cape of South Africa. A recent study of the genus revealed that *C. rosei* may in fact comprise several cryptic species, with a distinctive lineage potentially being confined to the Cape Peninsula. I test the hypothesis that breeding sites on the Peninsula form a single genetic lineage, but are distinct at a population level due to limited dispersal abilities and little if any gene flow. Aggregations at breeding sites were predicted to have high genetic diversity, as toads from a large area are thought to converge on a single site. In addition to directed field surveys to relocate historical breeding sites, phylogenetic and population genetic approaches were carried out using fragments of two mitochondrial markers (ND2 and 16S). To place the Peninsula sites within the greater *Capensibufo* phylogeny, sequenced gene fragments were analysed using parsimony and Bayesian inference methods. Population genetic structure was inferred through an Analysis of Molecular Variance (AMOVA) and standard and molecular diversity indices were utilised to estimate measures of genetic diversity.

As hypothesised, toads from the two surviving breeding sites on the Peninsula formed a single lineage that is sufficiently distinct to be given species status. There was no overlap in mtDNA haplotypes between individuals from the two sites, resulting in high values for both F_{ST} (79.77%) and Φ_{ST} (89.31%). This supports the hypotheses that gene flow between breeding sites is lacking and that they represent genetically distinct populations. Although these populations exhibited molecular differentiation, they lacked the expected high levels of

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genetic diversity. This may be an indication that these populations have experienced a reduction in effective population size (N_e), leading to increased inbreeding and thus reduced heterozygosity. The low levels of genetic diversity have major implications for these populations due to an increased vulnerability to extinction risk.

These results, coupled with an inability to re-locate other historically known breeding sites on the Cape Peninsula, make this newly recognised Peninsula endemic of national and international conservation priority and calls for the re-assessment of its IUCN Redlist status. Conservation effort should focus on the protection and expansion of the two known surviving populations and the patches of habitat on which they rely. Although translocation between the two populations is not advised due to their genetic distinctiveness, re-introduction at other suitable sites on the Peninsula to restore connectivity and gene flow is desirable.

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Chapter 1

General Introduction and Literature Review

Amphibians and the Biodiversity Crisis

The discipline of conservation biology emerged as a result of a growing concern for the current biodiversity crisis and a need to understand, quantify and alleviate this crisis (Soulé 1985). Of the rapid population declines and extinction of species being reported globally, perhaps some of the most alarming are those seen in global amphibian populations. The IUCN's Global Amphibian Assessment (GAA) reports that of the 5743 described species of amphibian over 43% are in a state of decline and 32.5% are globally threatened, with 7.4% of those listed as globally threatened being Critically Endangered (IUCN 2004). To further exacerbate the situation, these figures are thought to be underestimates, due to the large number of poorly studied species (Stuart et al. 2004). In comparison to other vertebrates, amphibians are proportionately more threatened than birds (12% threatened) and mammals (23% threatened) (Stuart et al. 2004; Rodrigues et al. 2006). Somewhat paradoxically, since these figures were published, a further 1320 amphibian species have been described, putting the global total at 6887 (AmphibiaWeb 2012). New species of amphibians are being named at a rate exceeding 2% per year (Wake and Vredenburg 2008). This rapid rate of discovery is not the result of taxonomic inflation, but rather due to increases in taxonomic work, the combined use of new molecular tools and traditional methods, and an increase in field exploration efforts (Vieites et al. 2009). Newly described amphibian species are often range restricted and isolated (Hanken 1999: Meegaskumbura et al. 2002; Köhler et al. 2005; Vietes et al. 2009), thus, while these discoveries may add to overall amphibian species richness, they may also result in an increased proportion of threatened amphibians.

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Natural or Anthropogenic?

Over the last four decades numerous reports have addressed the causes of the decline of amphibian populations and species. In the late 1970s, a seemingly simultaneous disappearance and decline occurred, with more than 500 amphibian species out of an estimated global total (at that time) of 4000 being reported extinct or declining (Barinaga 1990; Beebee and Griffiths 2005). Despite an upsurge of interest, the figures pertaining to declines in global amphibian populations were received, by some, with scepticism. Although not denying the role of habitat destruction and other anthropogenic effects, an exhaustive review of the literature concluded that declines of amphibian populations that are found in apparently pristine, isolated and protected areas cannot be separated explicitly from natural population fluctuations (Pechmann and Wilbur 1994). The study concluded that declines and disappearances in these situations represent a distinct phenomenon that goes beyond the general biodiversity crisis. It is important to note however, that although declines under such circumstances cannot be unequivocally linked directly to humans, the indirect influences of anthropogenic activities pose a real threat to amphibians, perhaps more so than for other classes of vertebrates.

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Amphibians are particularly vulnerable to environmental stressors due to their skin and eggs being permeable to water and electrolytes (Vitt et al. 1990; Blaustein et al. 1994a; Quaranta et al. 2009). Increased UV-B radiation as a result of stratospheric ozone depletion caused declines in some high altitude amphibian populations, through a slowing of growth rates and hampering of the immune system (Blaustein et al. 1994b; Blaustein and Johnson 2003; Pahkala et al. 2003; Marquis et al. 2008). Changes in patterns of rainfall and temperature (Davidson et al. 2002), the spread of epidemic diseases driven by global warming, (Pounds et al. 2006), sensitivity to air and water pollution (Carey et al. 2001; Davidson et al. 2002), and

changes in pH levels due to acid precipitation also have been identified as drivers of amphibian population declines (Vertucci and Corn 1996). In addition to being sensitive to a range of stressors, the complex, biphasic life histories of amphibians exposes them to both aquatic and terrestrial environmental threats (Vitt et al. 1990; Blaustein et al. 1994a; Blaustein 1994; Welsh and Ollivier 1998). Many amphibian populations have experienced "enigmatic declines", defined by Stuart et al. (2004) as declines that occur even when suitable habitat remains, for reasons that are not fully understood. However, although many areas where amphibian declines have occurred may appear to be pristine, the general degradation of the environment permeates beyond that of direct human activities such as habitat destruction, alien introductions and overexploitation. Numerous studies of amphibian losses and declines reveal that the underlying mechanisms are often complex, involving seemingly tenuous interactions among abiotic and biotic components, and that factors causing these declines are driving amphibian species toward extinction particularly rapidly (Blaustein and Kiesecker, 2002; Stuart et al. 2004). Amphibians contribute to the trophic dynamics of an ecosystem by acting as predators and prey; their loss may be felt at multiple trophic levels, impacting many other organisms within an ecosystem (Blaustein et al. 1994a; Blaustein et al. 2003).

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Irrespective of the differences of opinion regarding the causes of amphibian population declines, both believers and sceptics agreed there was a need for more data. The dearth of baseline data on the population dynamics of amphibians and the relatively brief and anecdotal nature of many studies led to difficulties in discerning between natural population fluctuations and human-induced declines (Barinaga 1990; Pechmann et al 1991; Pechmann and Wilbur 1994). A lack of substantiated data and thus the absence of appropriate mitigating management may have led to the demise of many amphibian populations and in

some cases entire species. The loss of the Golden Toad (*Incilius* (*Bufo*) *periglenes*) of Costa Rica, last seen in 1989, was originally ascribed to stochastic factors (Pounds and Crump 1994) until better data on climate changes and infectious fungal pathogens became available (Pounds et al. 1999; Cheng et al. 2011). The inability to elucidate proximate causes and discern between natural population fluctuations and anthropogenic driven declines may have obscured the species' vulnerability. However, even had the cause of the decline and eventual extinction of the Golden Toad been known, there would have been few conservation options for addressing its "enigmatic decline". Captive breeding is often the only option in these situations, which can yield poor results due to difficulties in maintaining populations in ex situ conditions (Stuart et al. 2004). However, conservation biology, being the crisis discipline that is, sometimes requires important tactical decisions to be made before there is confidence in the adequacy of the data (Soulé 1985).

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The Amphibian Crisis Today

With the advancement of biological research and the accumulation of better empirical evidence, our understanding of amphibian declines has improved. In a meta-analysis of 617 time series of population census data derived from 89 amphibian species (Green 2003), demonstrated that patterns of observed declines and increases were indicative of an overall population decline as opposed to natural population fluctuations. Houlahan et al. (2000), employing a strict selection criterion to avoid biases, summarised evidence for global amphibian population declines using data from 936 populations. They concluded that while there was considerable geographical and temporal variability, at a global scale amphibians had declined over the previous several decades and that they would continue to do so. Stuart et al. (2004), in a report on the status and trends of amphibian declines and extinctions, identified two important patterns of decline: (1) the geographical distribution of rapidly

declining species is non-random; and (2) the proportion of "enigmatic decline" species increases with increasing extinction risk; 92.4% of Critically Endangered amphibian species are thought to be "enigmatic decline" species. Based on a global data set of 5,527 amphibian species, Hof et al. (2011) attempted to elucidate, how the spatial interactions of three important threats (climate change, land-use change and the fungal disease chytridiomycosis) could affect global amphibian diversity between a baseline period (1980) and late in the present century (2080). Among other things, the study revealed a positive pattern between land-use change, climate change and predicted amphibian declines, highlighting the fact that there is a synergy between these two possible causes of decline. Interestingly, it was also found that the association between the projected occurrence of disease and climate change or land-use was weak, emphasising the potential for 'silent extinctions' away from regions of high anthropogenic impact.

Despite these somewhat belated advancements, there is a general consensus among herpetologists that there are still significant gaps in our knowledge. Key among these is the need to obtain more long-term quantitative data in order to further our understanding of the global amphibian crisis and to identify the proximate causes of declines. In the absence of long term data, biologists are frequently forced to use information on historical distributions and abundances garnered from museum records or natural-history databases, as often these records are the only historical ecological information on a species (Kress et al. 2001). Although such data typically lack information regarding observed absences, historical presences can aid by directing current search efforts (Skelly et al. 2003). However, it should be noted that the often *ad-hoc* nature of museum collections, with large gaps in temporal and spatial information, may lead to the misinterpretation of a species persistence and/or distribution (Ponder et al. 2001). The complexity of many amphibian declines means that

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advocating a single stressor is typically too simplistic; solving this issue requires an interdisciplinary approach, drawing upon expertise from a variety of fields, including conservation genetics, community ecology, immunology and biogeography (Blaustein and Kiesecker 2002; Collins and Storfer 2003).

The Role of Population Genetics

Conservation biology, which roots itself in the small and declining population paradigms (Caughley 1994), is a multidisciplinary science, taking questions, techniques and methods from a broad range of fields (Soulé 1985). One such field is conservation genetics. The discipline's fundamental concern is the identification and protection of future evolutionary potential of threatened species through understanding the importance of genetic diversity in the avoidance of species extinctions (Frankham et al. 2002; Monsen and Blouin 2003). Conservation genetics provides empirical evidence that can aid in the designation of conservation effort, while affording conservation biologists the ability to extend the "time scale of concern" to enhance the chances of future persistence of threatened populations (Frankel 1974; O'Brian 1994). A primary component of the discipline is population genetics. Where conservation genetics is concerned with how loss of genetic diversity and changes in genetic structure may affect the long-term survival of a species, population genetics chiefly focuses on understanding the factors responsible for the origin and maintenance of genetic variation in populations (Page and Holmes 1998; Wan et al. 2004).

Although not always the case, the retention of genetic variation and associated evolutionary potential should be a key point in the conservation of populations and species. Genetic variation facilitates evolutionary change within wildlife populations, allowing them to evolve in response to environmental change. The level of genetic variation within a species

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represents a balance between mutation, drift, and natural selection (Frankham 1996; Templeton et al. 2001). In general, when a species' population decreases (either through range contraction or reduced abundance within its range), it loses some genetic diversity among populations, individuals or genes (Scribner et al. 2001). Such populations are potentially more vulnerable to extinction risk due to an inability to survive stochastic environmental events (e.g. disease or natural disasters), demographic stochasticity (e.g. random variations in sex ratio, mortality or reproduction), genetic effects (e.g. mutation accumulation, genetic drift or inbreeding depression) and isolation (Templeton et al. 2001; Wan et al. 2004). In addition, the loss of genetic variation due to a reduction in population size frequently is correlated with a decrease in fitness through genetic drift and inbreeding depression (e.g. decreases in reproductive fitness due to inbreeding depression; reductions in adaptive potential due to genetic drift) (Amos and Blamford 2001; Templeton et al. 2001; Reed and Frankham 2003). Indicators of fitness are strongly correlated with population levels of genetic diversity (e.g. larval growth rates in *Epidalea* (*Bufo*) *calamita*; Rowe et al. 1999; Rowe and Beebee, 2003). The effects of a loss of genetic diversity differ from species to species with some surviving with very little variation (e.g. the Wandering *Diomedea exulans* and Amsterdam *D. amsterdamenis* Albatrosses, see Milot et al. 2007). The loss of genetic variation may not impact a population's fitness in its current environment (e.g. Northern elephant seals *Mirounga augustirostris*; see Bonnell and Selander 1974) but it may reduce the ability to adapt to future environmental change (Lacy 1997). The task for conservation biologists is to assess current genetic variation and investigate how the loss of genetic variation may affect the future potential of that species, as well as suggesting how best to prevent or remedy this loss (Sherwin et al. 2000).

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The Role of Phylogenetics

Since the early days of conservation genetics in the 1970s the development of widely used genetic methods have provided important insights that can critically affected management decisions (Wan et al. 2004; Frankham 2010). Conservation genetics has the potential to enable the genetic management of small populations by maximising retention of genetic diversity and minimising inbreeding, and permits the resolution of taxonomic uncertainties and delineation of conservation units (Frankham et al. 2002). The identification of possible units for conservation corresponds with the classification of sets of populations that have a common recent history, among which population transfers may be performed (Taberlet and Bouvet 1994). Additionally, assessing the evolutionary relatedness among groups of organisms through phylogenetic techniques permits the identification of cryptic species, thus directing conservation effort and the allocation of resources (Vane-Wright et al. 1991; Faith 1992; Cook et al. 2008; Vieites et al. 2009). Prior to the advancement of molecular techniques, the inference of organismal phylogeny was largely informed by morphology, ontogeny, behaviour and geographic distribution. In some cases these methods have resulted in distinct forms being overlooked (Cronin 1993; Taberlet and Bouvet 1994; Page and Holmes 1998). However, the emergence of the molecular era has not meant that traditional taxonomic criteria have become obsolete; many of these techniques remain valid and are used in conjunction with molecular evidence. Morphological characters are commonly used as a second line of evidence when investigating the phylogeny of taxa (see Baker et al. 1998 for a review) and can yield similar results to molecular data (Shoshani et al. 1996; Titus and Frost 1996). Additionally, where molecular data are not available, such as where extinct taxa are concerned, studies may be forced to rely on morphological analyses (Holmes and Page 1998).

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Mitochondrial DNA (mtDNA) is suitable for resolving taxonomic uncertainties and has been extensively used in phylogenetic analysis (Wan et al. 2004). MtDNA can also be used to estimate the extent of genetic divergence among populations, as well as to recognise recent bottlenecks due to fragmentation and isolation (Avise et al. 1987). The haploid DNA, which is carried by the mitochondria in the cell cytoplasm, is well suited to track genealogies through time due to its maternal form of inheritance, virtual lack of recombination and relatively rapid mutation rate (Avise et al. 1987; Avise 1995; Page and Holmes 1998; Beebee 2005; FAO 2007). Vertebrate mtDNA accumulates point mutations at an average rate 5-10 times higher than nuclear sequences (Randi et al. 1994). Resolving taxonomic discrepancies is crucial in ensuring the effective decision making of conservationists and wildlife managers. Without the utilisation of molecular approaches, we run the risk of condemning unrecognised species to extinction through, for example, the misidentification of an apparently widespread and low-risk species which may, in reality, comprise a complex of distinct taxa (Wan et al. 2004).

Whilst the use of mtDNA in the identification of genetically distinct groups is a commonly accepted practice, the associated neutrality of mtDNA and thus its applicability to the prediction of extinction risk and adaptation in the face of environmental change remains relatively unclear. Deviations from a strictly neutral model of evolution have been found in a variety of organisms (Haddrill et al. 2008; Ho et al. 2005; Rand 2001; Barton 1998; Charlesworth et al. 1993). Other studies suggest that neutral marker genes are generally uninformative when it comes to making inferences about the potential for adaptation (Reed and Frankham 2001; McKay and Latta 2002; Knopp et al. 2007). Therefore, to fully understand a population's vulnerability and adaptive potential, relying on mtDNA alone requires some caution. It is clear, however, that mtDNA can aid in our understanding of

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other processes that affect a population's fitness and thus its risk of extinction. Gene flow patterns, for example, can affect genetic structure and diversity, population size and thus long-term survival (Whitlock and McCauley 1999). Similarly, population size has been positively and significantly correlated with population fitness and thus the future adaptability, as discussed above.

Defining Units for Conservation

Identifying units for conservation has received a considerable amount of attention and stimulated much debate among conservation biologists. Ideally the entire species unit should be conserved, but managing and conserving all populations within a species is seldom feasible due to constraints in funding, land management, political boundaries and other social issues (de Guia and Saitoh 2007). The evolutionary significant unit (ESU), conceptualised by Ryder (1986), was developed to provide an objective approach to the prioritisation of units for protection below the species level by using information on the evolutionary history of populations (Vogler and DeSalle 1994). Since its formulation the concept has been the subject of much discussion among conservation biologists, systematists, and evolutionary biologists (Moritz 1994; Vogler and DeSalle 1994; Pennock and Dimmick 1997; Waples 1998; Dimmick et al. 1999; Paetkau 1999; Crandall et al. 2000; Fraser and Bernatcher 2001; de Guia and Saitoh 2007). Currently, although not without its critics, the most widely accepted definition is that ESUs are "geographically discrete populations which have evolved separately for a substantial period of time, being reciprocally monophyletic at mitochondrial DNA, and showing significant frequency difference of nuclear alleles" (Motitz 1994, p. 373). Such groups are considered to be on different evolutionary trajectories.

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Although Moritz's (1994) definition affords the protection of populations that display significant molecular differences, its reliance on neutral genetic variation has been questioned (Crandall et al. 2000; Fraser and Bernatcher 2001; de Guia and Saitoh 2007). De Guia and Saitoh (2007), in an extensive review of the literature concerning the practical application of the ESU concept, argue that the designation of such units for conservation should take on a more holistic form through the integration of neutral genetic variation and adaptive variation. Offering various examples, they suggest that an exclusively phylogenetic approach can lead to the omission of potential ESUs and recommend that ecological based traits be consider due to the possibility that they could be the driving force for selection, having the evolutionary potential to lead to genetic divergence. It should be noted, however, that when populations are small and/or rare, it is difficult to obtain the required ecological information to evaluate more robust definitions of ESUs. The authors concluded that where sufficient ecological data are lacking, the identification of "molecular ESUs" through the use of mtDNA should act as a practical framework and a starting point in identifying what they term a "full ESU", where the unit fulfils the original concept whilst reflecting true evolutionary variations.

Although theoretically sound and having the advantage of being qualitative rather than quantitative (Moritz 1994; Moritz 1995), the ESU concept has also been suggested to be overly restrictive due to it requiring a level of phylogenetic distinctiveness that is only achieved through long-term isolation (Moritz 1994; de Guia and Saitoh 2007). Additionally, nested units of diversity may be overlooked when the criterion of reciprocal monophyly is not met (Moritz 1994). Populations which reveal less phylogenetic separation than reciprocal monophyly have been defined as management units (MUs) (Moritz 1994). The MU is intended to be a level of conservation unit below that of the larger ESU (Wan et al. 2004), representing sets of populations that are currently demographically independent, with the

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degree of connectivity between populations being low enough to permit separate management (Moritz 1995; Palsbøll et al. 2007). The criterion most commonly used to delineate MUs is "populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of the alleles" (Moritz 1994, p. 374). Where the ESU criterion is concerned with the long-term management of historically isolated sets of populations that represent evolutionary unique entities, the MU focuses on contemporary population structuring and short-term monitoring (Moritz 1995). By determining recent genetic structure, dispersal and migration patterns in current fragmented populations, MUs identify entities for monitoring the effectiveness of management and the impacts of anthropogenic activities (Wan *et* al. 2004; Palsbøll et al. 2007; Schwartz et al. 2007).

Study Species: Rose's Mountain Toad Capensibufo rosei

Due to their limited dispersal abilities and the strong philopatry, many amphibians occur in distinct populations often with unique genetic identities. This makes them good candidates for conservation genetic studies and the application of conservation units. However our knowledge of amphibian evolutionary history lags behind that of amphibian species diversity (Jehle and Arntzen 2002; Beebee 2005; Frost et al. 2006). Although an increasing number of genetic studies have shown that large bufonids are wide-ranging (van Bocxlaer et al. 2009; van Bocxlaer et al. 2010), with the accompanying potential for high gene flow, very little information is available on the small, range-restricted members of the family whose conservation status is of increasing concern (Tolley et al. 2010).

Capensibufo Grandison, 1980 (Anura: Bufonidae) is a genus of African dwarf toad that consists of only two species, *Capensibufo rosei* and the allopatric *C. tradouwi*. Both species

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are confined to isolated, mountain plateaux in the Cape Fold Mountains of South Africa (Tolley et al. 2010). The genus is endemic to undisturbed mountain fynbos in the winter-rainfall region of the Western Cape Province with a limited and patchy distribution restricted to the Cape Peninsula and mountains southwest of the geographical barrier formed by the Breede River valley (Grandison 1980; de Villiers 2004). Breeding is dependent on sufficient rainfall occurring to form small, shallow, ephemeral pools of water. The vegetation surrounding these breeding pools is commonly dominated by restios (de Villiers 2004). Such wetlands are suitable for amphibians like *C. rosei* because they tend to lack predatory fish and birds typically associated with larger, permanent water bodies (Branch and Harrison 2004). A breeding site can consist of one or more small pools in a seepage area that itself may not exceed 100 m^2 (de Villiers 2004).

Capenisibufo rosei is categorised on the IUCN Red List as Vulnerable in view of its Extent of Occurrence (EOO) of 6526 km^2 and Area of Occupancy (AOO) of only 130 km^2 with a continuing decline in the AOO, quality of habitat and the number of subpopulations (SA-FRoG and IUCN 2011). Major threats to the persistence of *C. rosei* are reduced rainfall due to climate change, invasive alien vegetation, increasingly frequent fires, plantations, wildflower farming, afforestation, alteration of drainage patterns, building developments (e.g. road and dams), and urban development on the southern Cape Peninsula resulting in degradation or loss of habitat (de Villiers 2004). Perhaps the most serious threat to *C. rosei*, as with many South African anurans, is that of spreading invasive alien vegetation (de Villiers 2004; Angulo et al. 2011; Measey 2011; Measey et al. 2011a; 2011b). The encroachment of invasive plants threatens many species by reducing groundwater levels and increasing the risk of fire. This is particularly so in the fire-driven fynbos biome where invasive woody plants increase fuel loads and therefore fire intensity, from which many

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threatened amphibians struggle to recover (Branch and Harrison 2004; Measey 2011). While most *C. rosei* habitat is in pristine mountain areas, habitat degradation has led to >20% habitat loss over the last 30 years (de Villiers 2004).

Amphibians, like *C. rosei* tend to be overlooked in conservation planning due to their cryptic nature, a lack of data and because they use shallow, often small bodies of water for breeding that are typically ephemeral; this type of wetland tends to be ignored during spatial planning exercises rather than being recognised as habitats performing a vital function in ecosystems (Branch and Harrison 2004). An unusual characteristic of *C. rosei* is the absence of middle ear elements (Grandison 1980) and it appears to be the only southern African amphibian that lacks a voice. This, together with its montane distribution, small size, densely vegetated habitat and restricted breeding season, has resulted in a paucity of distribution records (Minter et al. 2004). It is possible that additional populations await discovery, but it is apparent that the species has disappeared from several known locations on the Cape Peninsula, suggesting that local extinctions have occurred. This is of particular concern because the disappearance of populations can be the prelude to species extinction (Ceballos and Ehrlich 2002). The possible loss of the species at these locations coupled with the already small AOO, and the species' Vulnerable status calls for the prioritisation of additional studies of *C. rosei*. The national strategy for the conservation of amphibians in South Africa (Measey et al. 2011a) states that additional work on this cryptic species is a high priority, with urgent need for phylogeographic and other systematic work in addition to the identification of management units.

Declines in global amphibian populations are predicted to accelerate in the twenty-first century with the greatest proportions of species negatively affected by climate change and

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land-use projected to be found in Africa, parts of northern South America and the Andes (Hof et al. 2011). Despite this, most African amphibians remain poorly studied in comparison to those of North and South America, Southeast Asia and the Malay Archipelago (Blackburn 2008). With more than one third of all known species of amphibian in a state of decline (Stuart et al. 2004; Wake and Vredenburg 2008) it is important to prioritise those species and populations that are at highest risk of extinction by delineating units for conservation and through understanding the underlying drivers of these often 'enigmatic declines'. Through the utilisation of historical records, phylogenetic techniques and population genetic analyses this study investigates the current distribution of *C. rosei* on the Cape Peninsula and assesses its vulnerability to extinction risks, whilst identify units for conservation priority and offering potential management guidelines.

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AIMS AND HYPOTHESES

A recent phylogeny of *Capensibufo* suggests that it may contain more than the two currently-recognised species, with *C. rosei* comprising multiple lineages and the Cape Peninsula possibly representing an independent lineage (Tolley *et al.* 2010). However, this conclusion was based on only two individuals from a single breeding site in the Silvermine section of Table Mountain National Park, above Muizenberg. The primary concern of my study is to test whether toads at all breeding sites* on the Peninsula form a monophyletic clade and if so, whether these breeding sites have significant population structure representative of genetically distinct populations. Additionally, levels of neutral genetic variation are assessed to evaluate the possible effects of isolation and declining population size on adaptive potential at each breeding site. These aims are addressed by testing the following hypotheses:

1. Breeding sites on the Cape Peninsula form a single genetic lineage.
2. Gene flow between sites is low due to limited dispersal abilities.
3. Breeding sites are distinct at the population level†.
4. Toads breeding at a given site have high genetic diversity, as animals from a large area converge on a single site.

In addition, this study collates historical records to direct search effort to assess whether toads remain active at historical sites on the Cape Peninsula. Where breeding sites appear to have gone extinct, I consider the possible drivers of these losses. However, the main conservation

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* From this point forward, the aggregation of toads at a particular location on the Peninsula will be referred to as a 'breeding site', representing not a geographical location but rather a biological entity.

† If genetic analyses reveal these 'breeding sites' are distinct at a population level, then the word 'population' will be used from that point forward.

priority is to resolve the taxonomic status of the Cape Peninsula population of *C. rosei* and to test for population structure and genetic diversity within surviving breeding sites.

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Chapter 2

Phylogenetics, Population Genetics and Historical Records of *Capensibufo rosei*

INTRODUCTION

With the suggestion that we are currently experiencing the sixth mass extinction, the decline of amphibians has received much attention over the past two decades (Wake and Vredenburg 2008). Amphibian populations worldwide are experiencing declines, with more than one third of all known species being in a state of decline and proportionately more amphibian species being at risk of extinction than those of any other taxon (Houlanhan et al. 2000; Stuart et al. 2004; Rodrigues et al. 2006). Elucidating the proximate cause of amphibian declines is known to be notoriously complex, a situation that is further exacerbated by a lack of baseline data. It is clear, however, that the mechanisms involved are multifaceted and rarely work in isolation. The ongoing debate around these declines is discussed in Chapter 1.

Rose's Mountain Toad, *Capensibufo rosei*, is one of two African dwarf toads that is endemic to the undisturbed mountain fynbos in the winter rainfall region of the Western Cape (de Villiers 2004). Through field studies over recent years, no evidence of breeding has been found at several former breeding locations on the Cape Peninsula, despite the presence of suitable habitat. This coupled with their restricted Area of Occupancy and Extent of Occurrence has resulted in *C. rosei* being categorised as Vulnerable on the IUCN Red List (SA-FROG and IUCN 2011). There are some uncertainties surrounding the taxonomy of *C. rosei* and its congener *C. tradouwi*, with lineages being found to be divergent across small geographic ranges due to their confinement to isolated seeps on mountain plateaux (Tolley et al. 2010). Isolated populations often pose a dilemma for taxonomists wishing to delineate

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species (Mayr 1942) as well as for conservation managers deciding how best to manage them (Measey and Tolley 2011). Genetic analysis of disjunct populations such as those found within *C. rosei* aids the decision making process by informing conservationists whether to manage them as discrete populations or if connectivity needs to be restored. This, along with an apparent disappearance of several breeding sites on the Cape Peninsula, makes *C. rosei* a prime candidate for phylogenetic and population genetic studies.

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MATERIALS AND METHODS

Historical Records and Directed Surveys

To direct search effort during the field component of this study, historical records from museums and related literature were compiled. Museum records were obtained by first consulting the literature and the Global Biodiversity Information Facility (GBIF) and then by requesting these records from the relevant institutions. Records from the Atlas and Red Data Book of the Frogs of South Africa, Lesotho, and Swaziland (Minter et al. 2004) were also included, as were field notes and anecdotal information from local herpetologists. Records were conciliated by sighting, i.e. if more than one record referred to the same sighting it was only included once in this study (Appendix A). In the field, teams of 1-8 people searched on 21 occasions at 12 known breeding sites on the Cape Peninsula (Figure 1) for evidence of breeding aggregations or individual toads. In order to maximise detection, the search component of this study started before breeding began at known locations in July 2011, continued throughout breeding and was concluded at the end of October 2011, after tadpoles had metamorphosed.

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Sampling Procedure and Laboratory Methods

Toe clips were taken from 60 *C. rosei* on five different occasions from two breeding locations Silvermine (n=31) and Cape Point (n=29). Samples were stored in 99% ethanol. Total genomic DNA was extracted using the DNeasy tissue kit (Qiagen©) following manufacturer's instructions and portions of two mitochondrial markers (NADH dehydrogenase subunit 2 (ND2) 535 bp and 16S Ribosmal RNA(16S) 508 bp) were amplified using the Polymerase chain reaction (PCR) in 25µL reaction volumes (Table 1).

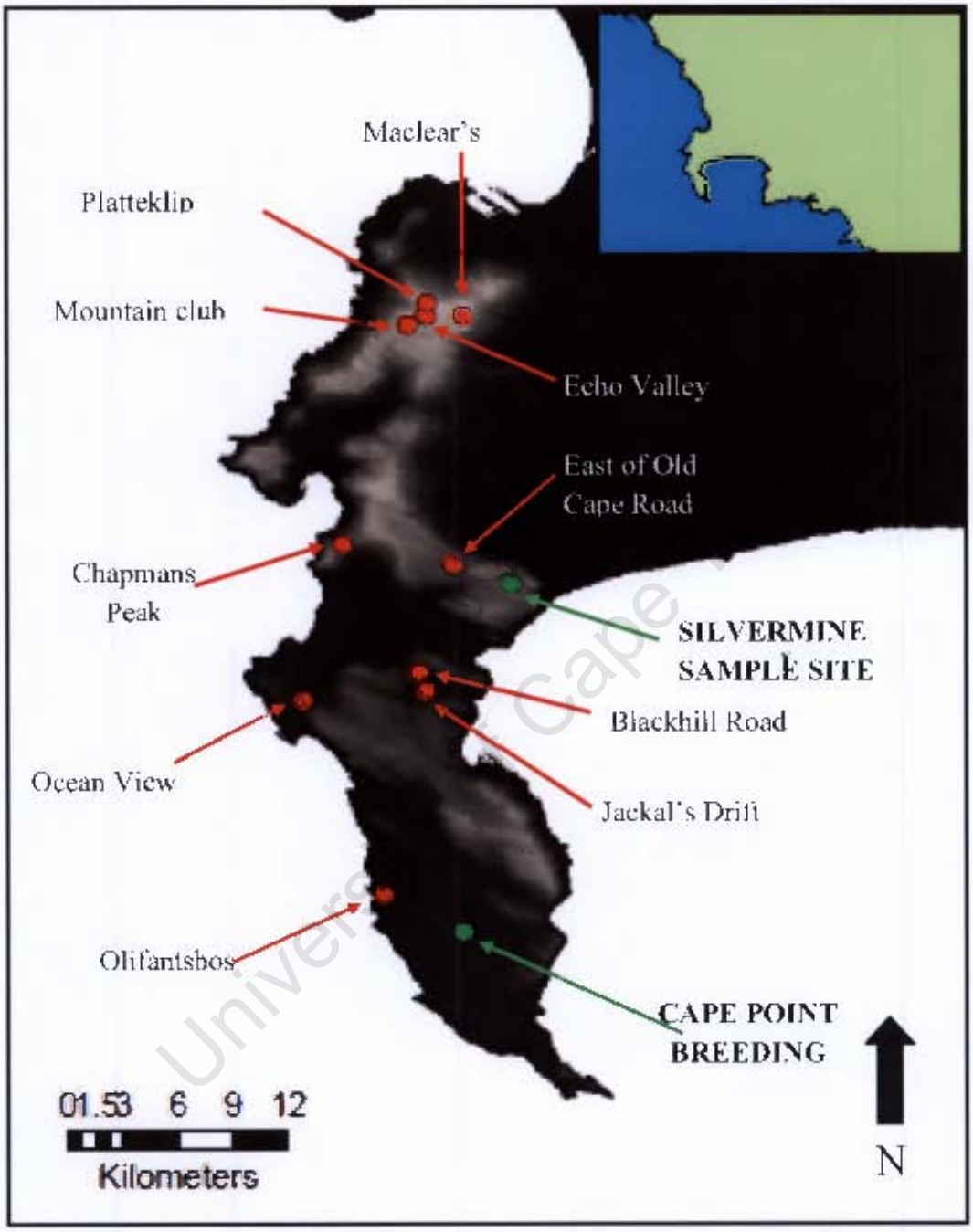

Figure 1: A map of the Cape Peninsula showing all sites where *C. rosei* has been recorded and the location of all breeding sites searched for during this study. Green points represent breeding sites that were re-located in 2011 and the red points represent breeding sites that were not been re-located.

Table 1: Conditions for PCR amplification at two mtDNA target regions.

Reagent	ND2	16S
Buffer (µL)	5	2.5
dNDPs (mM)	0.3	0.3
$MgCl_2$ (mM)	2.5	2.5
F Primer (µL)	0.3	0.3
R Primer (µL)	0.3	0.3
Taq (U)	0.25	0.2
DNA (µL)	2.0	2.0
Temperature (°C)	50-58	52/56

Undiluted DNA was used as template with concentrations of between 25 and 45 ng/µL. ND2 was amplified from vMet2 (5'-GCT AAA CAA GCT TTC GGG CCC ATA CC-3') to vTrp (5'-CTC CTG CTT AGG GCT TTG AAG GC-3') (Cunningham and Cherry 2004), using GoTaq DNA polymerase and 5 × colourless GoTaq Flexi Buffer (50mM Tris-HCl, pH8.5). Primers were chosen in line with previous studies on the *Capensibufo* genus (Tolley et al. 2010). 16S was amplified from 16Sa (5'-CGC CTG TTT ATC AAA AAC AT-3') to 16Sb (5'-CCG GTC TGA ACT GAG ATC ACG-3') (Palumbi et al. 1991) and 16SaL (5'-CGC CTG TTT ATC AAA AAC AT-3') to 16SbH (5'-CCG GTC TGA ACT CAG ATC ACG T-3') (Vences et al. 2005), using Super-Therm Taq polymerase and 1 × thermophilic Buffer (50mM KCl, 10mM Tris-HCl, pH 9).

The thermal profile used for both markers comprised an initial denaturation step of 94°C for 4 minutes, followed by 35 cycles of 30 seconds at 94°C (denaturation), 30 seconds at 50-58°C (primmer annealing), 1 minute at 72°C (extension), and a final extension step of 72°C for 8 minutes using Thermo Cycler 2720 (Applied Biosystems). 3 µL of amplified PCR product combined with 2µL of loading dye were electrophoresed on 0.8% agarose gels (100V for 25

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minutes) containing ethidium bromide and visualised using UV light. Successfully amplified PCR products were sent to Macrogen Inc., Korea for sequencing. In some cases when PCR products gave unreadable sequences, the above steps were repeated and new PCR products were again submitted.

Sequence Alignment and Editing

Multiple sequence alignments were generated by using Geneious Pro 4.8.5 (Drummond et al. 2010). Over evolutionary time, related DNA or amino acid sequences diverge through the accumulation of mutation events such as nucleotide or amino acid substitutions, insertions and deletions (Drummond et al. 2010). A multiple sequence alignment compares multiple related DNA or amino acid sequences in an attempt to determine regions of homology and can be used for many purposes including inferring the presence of ancestral relationships between the sequences (Drummond et al. 2010). A global alignment, which involves the alignment of more than two sequences using a heuristic approach, was chosen due to the relatedness of the sequences; if the relatedness of sequences had been unknown or shared only small regions of similarity, a local alignment would have been more appropriate (Drummond et al. 2010). When selecting the global alignment type, gaps at either end of the alignment were not penalised when determining the optimal alignment. Alignment parameters were set at 65% similarity, with a gap open penalty of 12 and a gap extension penalty of 3 (Dietz et al. 2011). Geneious then applied a progressive pairwise alignment algorithm (after Feng and Doolittle 1987). Progressive alignment algorithms generate fast and, in most cases, reasonably accurate results (Abecasis et al. 2007).

A visual sequence inspection was carried out to check for incorrect base callings. As algorithmic alignment does not necessarily retrieve the best alignment (Notredame 2002), it

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is essential to verify that the sequence data are aligned unambiguously and, if necessary, manually correct the alignment (Abecasis et al. 2007). ND2 sequences were translated to protein codons, and examined for the presence of stop codons. If a base could not be confidently identified across the majority of sequences it was coded as ambiguous and omitted from the analysis. Once sequence alignment was complete ND2 and 16S alignment blocks were converted to nexus files to conduct subsequent analysis.

Phylogenetic Analyses

To determine the taxonomic placement of the Cape Peninsula breeding sites, a data set with both ND2 and 16S was constructed using 5 individuals per site plus previously published sequence data from 30 individuals of the *Capensibufo* genus (including *C. tradouwi*), and 2 out-group taxa (*Vandijkophrynus angusticeps* and *Vandijkophrynus gariepensis*) (Tolley et al. 2010). Prior to phylogenetic analysis, jModelTest 0.0.1 (Posada 2008) was run for each marker to investigate the evolutionary model that best fit the data set. Models of nucleotide substitution allow for the estimation of probabilities of change between nucleotides along the branches of a phylogenetic tree (Posada 2008). These models also determine the way in which tree building programs calculate branch length (Hall 2011). To test which evolutionary model best explained the data, jModelTest employed a likelihood optimisation function using Phyml 2.4.4. (Guindon and Gascuel 2003). For both genes Akaike's information criteria (Akaike 1973) chose the TrN+G substitution model (Tamura and Nei 1993). When selecting models for phylogenctic analysis there is a trade-off between simple models that can be inaccurate representations of sequence change, and complex models which can be over-specific due to treating stochastic error in the data set as deterministic features of the evolutionary process (Cunningham and Cherry 2004). Thus when jModelTest

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selects a model, those with larger likelihoods are considered to be a better fit; however a penalty factor is incorporated for models that are parameter rich.

Phylogenetic analysis of 1043 characters from the two mitochondrial genes (ND2, 535 bp and 16S, 508 bp) was performed using Bayesian Inference (BI) in MrBayes 3 (Ronquist and Huelsenbeck 2003). The data were partitioned by gene and run independently to estimate the model parameters for each. Priors on the partitions were set using the model indicated in jModelTest. BI seeks the tree that is most likely given the data and the selected nucleotide substitution model (Hall 2011). Thus for both gene partitions, MrBayes was run specifying six rate categories, including rate variation among characters and estimations of nucleotide frequencies.

Markov chain Monte Carlo (MCMC) simulations were run twice in parallel with four independent chains (one cold, three heated) starting from different initial random trees, for 10 million generations. Chains were swapped using the MrBayes default setting; chain swapping ensures that the MCMC process does not get stuck at a local maximum (Hall 2011). Trees were sampled every 1000 generations after the first 3 million generations were removed as burn-in, excluding them from posterior probabilities and branch-length compilations. Removal of burn-in is necessary to exclude low-likelihood trees encountered near the beginning of the run when calculating the consensus tree. Burn-in was determined by examining when the standard deviation of split frequencies stabilised and whether the effective sample size > 200 for all parameters using Tracer 1.4.1 (Rambaut and Drummond 2007). A 50% majority rule tree was constructed and nodes with ≥ 0.95 posterior probability were considered supported.

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A parsimony search was also run, as an alternative tree building method, in PAUP*4.1b10 (Swofford 2002). A heuristic search was performed in order to choose the tree with the least number of evolutionary steps. Reliability of the tree and confidence in the nodes was assessed by 1000 bootstrap replicates, each with 50 random-addition replicates. In phylogenetics, reliability of a tree is fundamentally the probability that the members of a given clade are always members of that clade (Hall 2011). Here nodes with a bootstrap value of $\geq$ 75% (meaning that a particular node occurred in $\geq$ 75% of the bootstrap replicates) were considered to be supported.

Population Genetic Analyses

Population genetic methods were used to examine genetic structure between breeding sites within the Cape Peninsula. To identify how many haplotypes were present at each sample site, a search of redundant taxa was performed in MacClade 4.0 (Maddison and Maddison 2000). When analysing DNA sequence data in MacClade, the term taxa refers to the basic units with which characters are ascribed (Maddison and Maddison 2000). Therefore, in this case a taxon is representative of an individual toad and its associated DNA sequence. MacClade considers two taxa redundant if their states are absolutely identical (Maddison and Maddison 2000). Haplotype diversity (h) and nucleotide diversity (π) were estimated based on standard diversity indices and molecular indices using Arlequin 3.1.1 (Excoffier et al. 2005). Haplotype diversity is an analogous measure of heterozygosity. It represents diversity at a specific haploid marker and is a function of the number of different haplotypes and their frequencies in each group (Nei 1987). The more informative measure of nucleotide diversity is a function of genetic variation and takes into account the actual number of base changes between sequences (Page and Holmes 1998). Standard diversity indices compute several common indices of diversity, like the number of alleles, the number of segregating

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loci and the heterozygosity level, molecular diversity indices compute several indices of diversity at the molecular level (Excoffier and Heckel 2006).

Population genetic structure was inferred by computing a distance matrix and performing an Analysis of Molecular Variance (AMOVA), which was used to calculate *F*-statistics (F_{ST}) (Wright 1951) in Arlequin 3.1.1 (Excoffier and Heckel 2006). F_{ST} quantifies how genetic diversity is partitioned within and between populations or groups of populations by identifying the similarities in allele (haplotype) frequencies. If allele frequencies are similar, F_{ST} is low, and conversely, if allele frequencies are differ F_{ST} is high. Thus high levels of gene flow will result in little differentiation and a low F_{ST} value (Kronholm et al. 2010; Page and Holmes 1998,). F_{ST} is influenced by several evolutionary forces, such as mutation and migration (Excoffier and Heckel 2006). An analogous estimator to F_{ST}, Φ_{ST}, is a modification of the Fixation Index for molecular sequence data, which takes into account "distances" among alleles and reflects phylogenetic relationships among haplotypes (Holsinger and Weir 2009). When implementing the AMOVA approach, genetic structure is estimated using information on the allelic content of haplotypes as well as their frequencies (Excoffier et al. 1992). Levels of significance were determined through Monte Carlo resampling simulations, with 10,000 random non-parametric permutation replicates. The Tamura-Nei method with the gamma shape parameter ($\alpha = 100$) was used to generate the distance matrix. The Tamura-Nei method corrects for rate variability among sites whilst the shape parameter of the gamma function allows for unequal mutation rates among sites (Excoffier et al. 2005). Net sequence divergence (p distances) between the two populations was estimated in MEGA 4.0 (Tamura et al. 2011). In addition, relationships among haplotypes were examined with a median-joining network in Network 4.6.1.0 (Bandelt et al. 1999).

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RESULTS

Historical Sites and Directed Survey

A total 124 *Capensibufo rosei* records were collated from seven museums, 47 records from seven herpetologists' field notes and 10 anecdotal records from 18 sites in 12 general locations on the Cape Peninsula (Figure 1, Appendix A). Most historical observations occurred in August and September, which coincides with breeding time for this species, and span from 1901 to the present. There are also some negative observations in these historical records. There have been no observations made on Table Mountain since 1983, where it seems, from the records, that there were previously at least two well known breeding sites (Maclears Beacon and Platteklip Gorge). Similarly, several active breeding sites were reported up until the 1970's such as a site east of the Old Cape Road (Silvermine), at Olifantsbos (Cape Point) and another near Ocean View, Kommetjie. During 2011, over a period of 21 survey days at 12 historical locations, only two breeding sites (Steenberg Plateau, Silvermine and near Gilli Dam, Cape Point) were found to have adult toads, spawn and tadpoles. Seven of the 10 historical sites where *C. rosei* appears to have disappeared still have seemingly suitable habitat. The three exceptions are Jackal's Drift (developed into a residential area), Ocean View (infested by alien vegetation) and Platteklip Gorge on Table Mountain, which has been heavily impacted by tourism due to its location along a main footpath.

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Phylogenetics

Five individuals from each of the two Cape Peninsula breeding sites located were included in the phylogenetic analysis. Bayesian and parsimony methods both produced the same basic topology (Figure 2). *C. rosei* consists of four divergent clades (Figure 2) but the relationships between them were unresolved. Sequence divergences suggest geographic isolation between

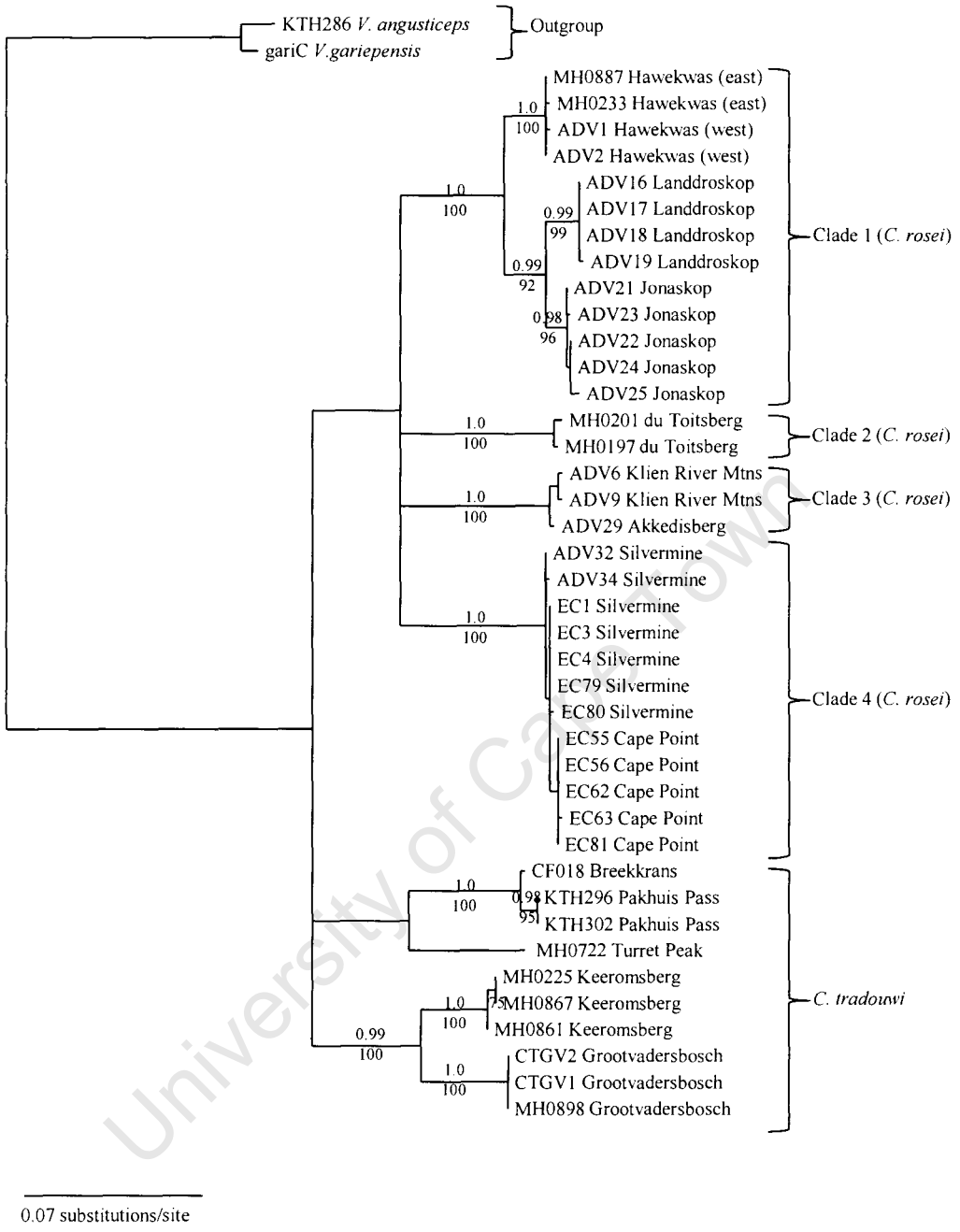

Figure 2. Bayesian consensus phylogram for *Capensibufo*, based on amplified fragments of 16S and ND2. Node support is shown by posterior probabilities above each branch, and parsimony bootstrap values below. Clades 1 through to 3 consists of populations located off the Peninsula, Clade 4 consists of the two breeding sites studied in this thesis, which are both located on the Peninsula.

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the four clades, a pattern similar to that seen in *C.tradouwi* (Tolley et al. 2010). The two breeding sites on the Peninsula form a single monophyletic lineage (Table 2).

Population Genetics

Sequencing of 535 base pairs of the ND2 mitochondrial gene revealed five haplotypes among the 62 individuals analysed for the two breeding sites (Silvermine n=33, Cape Point n=29, Table 3). There were no haplotypes shared between sites, suggesting restricted gene flow. The Silvermine sample contained three haplotypes, with haplotype 1 accounting for 82% of individuals sequenced. The Cape Point population consisted of only two haplotypes, with haplotype 3 found in all but one individual (97%). This pattern is reflected in the haplotype network (Figure 3) in which no haplotypes were shared across the two breeding sites and the number of haplotypes was greater at Silvermine. Haplotype (h) and nucleotide (π) diversity was notably greater for the Silvermine breeding site than that at Cape Point (Table 4).

The analysis of molecular variance (AMOVA) for haplotype frequencies (F_{ST}) indicated that there was a significant amount of genetic variation among the two breeding sites as opposed to within (79.77% of the variation occurring among populations, P<0.001; appendix B). This suggests that there is a lack of gene flow between the two breeding sites and that these sites are strongly differentiated from one another thus representing genetically distinct populations. The AMOVA resulted in an equally significant high pairwise Φ_{ST} value for variation among populations (89.31% of the variation occurring among populations, P<0.001) (appendix B). This again indicates structure at the population level. Although there is significant population structure, net sequence divergence (p distance) between the populations was low at 0.04 % for ND2 and 0.00 % for 16S. In comparison, p distances between the Silvermine and Cape Point populations and those off of the Peninsula range from 9-12% for ND2 and 2-4% for 16S (Table 2).

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Table 2. Net sequence divergence (p distances) for Rose's Mountain Toad, *Capensibufo rosei* and Tradouw's Mountain Toad, *Capensibufo tradouwi* for16S (upper matrix) and ND2 (lower matrix).

Species	Site	Hawkewas (east)	du Toitsberg	Breekkra-ns	Turret Peak	Keeromsbe-rg	Grootvade-rsbosch	Hawekwas (west)	Klien River Mtns	Landroskop	Jonaskop	Akkedisberg	Pakhuis Pass	Silvermine	Cape Point
		rosei	*rosei*	*tradouwi*	*tradouwi*	*tradouwi*	*Tradouwi*	*Rosei*	*rosei*	*rosei*	*rosei*	*rosei*	*tradouwi*	*rosei*	*rosei*
rosei	Hawekwas (east)	*NA/0.003*	0.037	0.044	NA	0.051	0.051	0.000	0.039	0.014	0.008	0.037	0.048	0.035	0.035
rosei	du Toitsberg	0.078	*0.002/0.004*	0.036	NA	0.035	0.031	0.037	0.028	0.027	0.028	0.027	0.041	0.029	0.029
tradouwi	Breekkrans	0.123	0.116	*NA/NA*	NA	0.036	0.038	0.044	0.040	0.036	0.034	0.042	0.002	0.038	0.038
tradouwi	Turret Peak	0.124	0.118	0.099	*NA/NA*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
tradouwi	Keeromsberg	0.123	0.109	0.089	0.112	*0.002/0.002*	0.010	0.051	0.047	0.042	0.043	0.045	0.039	0.045	0.045
tradouwi	Grootvadersbosch	0.121	0.120	0.118	0.114	0.062	*0.000/0.000*	0.051	0.047	0.042	0.043	0.045	0.040	0.043	0.043
rosei	Hawekwas (west)	0.000	0.082	0.124	0.122	0.126	0.120	*0.000/0.000*	0.039	0.014	0.008	0.037	0.048	0.048	0.035
rosei	Klien River Mtns	0.078	0.091	0.108	0.112	0.107	0.118	0.078	*0.002/0.002*	0.030	0.032	0.006	0.042	0.035	0.036
rosei	Landroskop	0.043	0.095	0.112	0.121	0.119	0.116	0.044	0.085	*0.001/0.000*	0.006	0.028	0.040	0.026	0.026
rosei	Jonaskop	0.039	0.096	0.112	0.124	0.114	0.110	0.041	0.087	0.019	*0.002/0.002*	0.030	0.038	0.027	0.027
rosei	Akkedisberg	0.083	0.095	0.114	0.120	0.119	0.127	0.082	0.000	0.088	0.090	*NA/NA*	0.044	0.037	0.037
tradouwi	Pakhuis Pass	0.126	0.117	0.009	0.104	0.097	0.120	0.128	0.112	0.116	0.117	0.119	*0.000/0.000*	0.042	0.042
rosei	Silvermine	0.101	0.099	0.104	0.119	0.112	0.124	0.104	0.093	0.097	0.103	0.098	0.107	*0.001/0.001*	**0.000**
rosei	Cape Point	0.104	0.099	0.099	0.120	0.109	0.123	0.107	0.096	0.095	0.101	0.101	0.104	**0.004**	*0.001/0.000*

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Values on the diagonal (italicised) reflect within-site p distances (16S/ND2). Values in bold show estimates for the two sites in this study (Silvermine and Cape Point). NA, not estimated for within-site divergences (only one sample at a site), or because of non-amplification of the 16S fragment (Turret Peak). Apart from Silvermine and Cape Point, all other samples for both *C. rosei and C. tradouwi* were located on the Peninsula (see Tolley et al. 2010 for point localities). Sequence data for all sites other than Silvermine and Cape Point were provided by Dr. Krystal Tolley.

Table 3. Haplotype frequency and distribution for Rose's Mountain Toad, *Capensibufo rosei*, in Silvermine and Cape Point using amplified fragments of ND2. GenBank accession numbers are yet to be obtained.

Haplotype	Frequency	Silvermine	Cape Point	GenBank
Hap_1	27	27	0	TBO
Hap_2	4	4	0	TBO
Hap_3	28	0	28	TBO
Hap_4	2	2	0	TBO
Hap_5	1	0	1	TBO
Total	62	33	29	TBO

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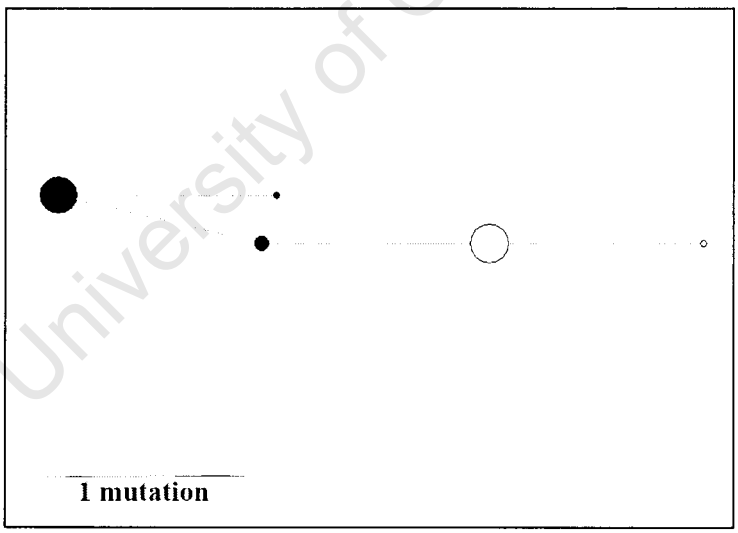

Figure 3. A median-joining network of haplotypes (ND2 mitochondrial gene) for Rose's Mountain Toad, *C. rosei*. Haplotypes are represented by circles and show the proportion of toads from Silvermine (black) and Cape Point (white). The area of the circle is proportional to the number of individuals with that haplotype, and the length of the connecting lines is proportional to the number of base changes between haplotypes.

Table 4. Number of individuals (*n*), number of haplotypes (*h*), and molecular diversity measures (π) for the two Cape Peninsula populations of Rose's Mountain Toad, *Capensibufo rosei* and a selection of other anurans, with various geographic ranges and life history traits. Where more than two populations were studied in a publication, only the lower and upper range values are displayed here.

Species	Population location	*n*	Haplotypes	Gene diversity (*h*)	Nucleotide diversity (π)	Reference
Capensibufo rosei	Silvermine, South Africa	33	3	0.322 ± 0.097	0.00063 ± 0.00071	This study
Capensibufo rosei	Cape Point, South Africa	29	2	0.069 ± 0.063	0.00012 ± 0.00030	This study
Eleutherodactylus stejnegerianus	Las Cruces, Costa Rica	8	2	-	0.0000 ± NA	Crawford 2003
Eleutherodactylus stejnegerianus	Palmar Norte, Costa Rica	8	4	-	0.00749 ± NA	Crawford 2003
Thoropa miliaris	N. Atlantic Coast Forest, Brazil	9	9	1.00 ± 0.052	0.038 ± 0.020	Fitzpatrick et al. 2009
Thoropa taophora	Juréia, Brazil	6	5	0.933 ± 0.122	0.0017 ± 0.0011	Fitzpatrick et al. 2009
Amietophrynus pantherinus	East of False Bay, South Africa	43	3	0.495 ± 0.048	0.00063 ± 0.00061	Measey and Tolley 2011
Amietophrynus pantherinus	Cape metro area, South Africa	110	8	0.728 ± 0.027	0.00128 ± 0.0006	Measey and Tolley 2011

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DISCUSSION

Despite dedicated search efforts over recent years, only two known populations of *Capensibufo rosei* on the Cape Peninsula have been re-located (Silvermine and Cape Point). This suggests that many breeding populations have become extinct. The apparent decline in populations, coupled with its already narrow distribution and specialised habitat requirements, makes this species of great conservation concern and indicates that the current Vulnerable classification for the species may be inadequate. Currently the species (*sensu lato*) is known from only eight locations two on the Cape Peninsula and six in the fold mountains east of Cape Town (Appendix C). However, this study has shown that the two populations on the Peninsula form a single genetic lineage that is quite distinct from the three lineages found farther east. Although the Peninsula populations form one genetic lineage, high fixation index values and a complete lack of shared haplotypes between sampled individuals indicates that these breeding sites have structure at a population level and that there is limited gene flow between them. Worryingly, both populations exhibit low molecular diversity, suggesting that their population size may be making what is possibly the last two extant populations of a Peninsula endemic vulnerable to stochastic events.

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Phylogenetics

In order to address the hypothesis that populations on the Cape Peninsula form a single genetic lineage (hypothesis 1), this study utilised phylogenetic analysis of two mitochondrial markers (ND2 and 16S). Despite the propensity for *Capenisibufo* lineages to display significant divergences across small geographic distances (Tolley et al. 2010), the two sampling sites located on the Cape Peninsula, separated by a distance of ~20km, were not separate lineages. The phylogenetic tree produced for *Capensibufo* suggests the presence of four distinct clades of *C. rosei* (Figure 2) with the two Peninsula populations (Silvermine and

Cape Point) forming one distinct evolutionary lineage (clade 4 in Figure 2). This conclusion is further supported by the low net sequence divergence exhibited between Silvermine and Cape Point (ND2 = 0.04%; 16S = 0.00%), values that are usually seen within rather than between species (Cunningham and Cherry 2000; Vences et al. 2005). Both sample sites on the Peninsula are separated from the eastern populations by 9-12% sequence divergence for ND2 and 2-4% sequence divergence for 16S (Table 4). Such values are typical of the differences between rather than within species (Vences et al. 2005; Wu et al. 2009) suggesting that the Cape Peninsula population is best treated as a separate species.

Molecular Population Structure

Of the individuals sampled at each breeding site, an examination of population level variation showed no overlap in haplotypes (Table 2, Figure 3). Thus, contemporary gene flow between the two sites is unlikely, allowing the acceptance of hypothesis 2. This lack of dispersal between populations may have been exacerbated by the apparent loss of other Peninsula populations; if other intervening populations are located a clinal pattern may exist, with haplotypes common to each population occurring at intermediate frequencies (Page and Holmes 1998). Differing haplotype frequencies and a high incidence of private haplotypes (haplotypes occurring in only one population), as seen here, are indicative of physical geographic separation or low gene flow for an extended period of time, where by stochastic lineage sorting can eliminate shared haplotypes over time, a process that is enhanced in small populations (Avise et al. 1987; Avise 1992). Where populations are not panmictic, one would expect to see the accumulation of small mutations that delineate a lineage on a unique evolutionary trajectory (Wright 1951; Smith and Green 2004). Therefore, the geographic pattern of genetic variation is influenced by a combination of population structure and population history. The behaviour and biology of amphibian's means that they are often

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restricted to relatively isolated populations associated with discrete habitats such as breeding ponds (Beebee 2005; Jehle et al. 2005) and therefore often exhibit strong population structuring over relatively small spatial scales.

The high degree of isolation among the two sites as indicated by the geographic structure in the distribution of haplotypes (Figure 3) is further supported by the AMOVA, where significantly high values for both F_{ST} and Φ_{ST} indicate that most of the genetic diversity is partitioned among the two populations, rather than within (Appendix B). The results could be an indication that recent human activities may have led to the loss of connecting populations between these two populations, thus enhancing drift and diminishing gene flow. Consequently the two populations may then follow the classic allopatric model of speciation, whereby genetic variation, the raw material for speciation, results in them being on different evolutionary trajectories (Page and Holmes 1998). However, it seems more likely that with the suspected loss of intervening breeding sites suppressing immigration, the two populations may disappear before they can diverge further (Templeton et al. 2001). In concurrence with hypothesis 3, it is apparent that through lineage sorting over time, these two breeding sites are representative of genetically distinct populations that have reached a stage of reciprocal monophyly, sharing no haplotypes and being genetically more similar within than among populations.

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Genetic Diversity

Gene diversity (h) and nucleotide diversity (π) for the two Cape Peninsula populations of *C. rosei* are low in relation to other species of anurans sampled for the same gene, with the Cape Point population displaying lower diversity for both indices than the Silvermine population (Table 3). During the field survey, the numbers of tadpoles and quantity of spawn at the

Cape Point site was significantly lower than that at Silvermine, additionally no metamorphs were observed at Cape Point. This may have been a natural fluctuation in population size, but with the apparent loss of other Peninsula populations over the past 40 years, it raises concerns over the long-term viability of the Cape Point population. Declining populations often display low genetic variability, where a reduction in the effective population size (N_e) causes only a small number of haplotypes to persist (Page and Holmes 1998; Allendorf and Luikart 2007). A reduction in population size can be of further detriment due to an increase in the amount of inbreeding, which in turn reduces the heterozygosity of a population. Additionally, genetic drift, which affects all finite populations and results in the fixation or extinction of certain alleles, can contribute to a reduction in genetic variation. As N_e decreases, the rate of fixation of alleles through genetic drift increases, thus becoming more pronounced in smaller populations (Ghiselin 2002). Levels of heterozygosity have been linked directly to fitness and recruitment in some anuran populations, where lower than average hatch, larval growth and larval survival rates were attributed to a loss of genetic variation (Rowe et al. 1999; Semlitsch et al. 2000; Rowe and Beebee 2003; Anderson et al. 2004).

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Genetic diversity is key to the long-term survival of populations and the importance of preserving genetic variation within populations has been recognised for influencing short-term population viability as well as maintaining a population's ability to adapt to environmental change (Frankham 1995), although the role of neutral genetic variation in this respect remains a point of contention (Knopp et al. 2007). Due to the lack of temporal genetic data for the two populations and insufficient knowledge concerning the ecological based traits of the species, it is only possible to infer the processes that are contributing to the low genetic variation, although since no process acts in isolation it is likely that there is more

than one at work. However, in comparison to other anurans, it is clear that the genetic diversity of *C. rosei* is low, especially at the Cape Point breeding site. The lack of genetic diversity is of major concern for this newly recognised Peninsula endemic and leads to the rejection of hypothesis 4, which expected high genetic diversity due to the convergence of toads from a large area on a single breeding site.

Resolution of Taxonomic Uncertainty

The results suggest that the evolutionary history of *C. rosei* is more complex than previously recognised, and that the species may in fact be comprised of a number of undescribed cryptic species. A value of 3% sequence divergence for 16S has been advocated for the identification of cryptic diversity within anurans (Fouquet et al. 2007). This value was suggested as an optimal threshold, being low enough to eliminate the risk of missing potential species, but high enough to ensure against the misidentification of conspecific lineages as species. These conclusions, however, were based on Neotropical anurans, where genetic diversity between species is higher than that of their temperate counterparts. Combining the data provided here with our current knowledge on the geographic distribution and dispersal abilities of *C. rosei*, it is apparent that there are complex phylogeographic relationships amongst populations in clades 1, 2 and 3 (Figure 2) and that the taxonomy of this species needs to be re-examined. However, further genetic and morphological analysis would be required for a comprehensive assessment. Regardless, it is possible to confidently recognise that together the Cape Peninsula populations form a lineage that is sufficiently distinct to be given species status. As the species was described from a holotype collected on the Cape Peninsula, the specimens found farther east are placed in a state of *incertae sedis* while those on the Peninsula will remain *Capensibufo rosei*. In light of the evidence presented here, a re-

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evaluation of *C. rosei sensu stricto* for IUCN Redlist status will undoubtedly lead to the species being categorised as Endangered or possibly even Critically Endangered.

Conservation Implications

In a genetically and geographically discontinuous species like *C. rosei sensu stricto* it is possible that each isolated population may represent an emergent historical entity. This is certainly true of the two populations observed on the Peninsula. The strong genetic population structure, apparent lack of gene flow and high genetic differentiation found between the populations of the Peninsula lineage highlights the need to consider the management of this species on a more discrete scale. Being historically isolated, geographically distinct and reciprocally monophyletic at the mitochondrial level means the Peninsula lineages fulfil the criteria of being treated as separate management units (MUs). On a less discrete scale, it is reasonable to suggest, even in the absence of nuclear markers, that the Peninsula populations should encompass a separate evolutionary significant unit (ESU) to those found farther east.

Given that the habitats where these populations occur do not differ drastically between sites and that both populations may be threatened by similar factors, aside from some level of human disturbance at the Silvermine site due to the presence of a frequently used footpath, the two MUs could be subjected to similar conservation measures. However, it should be noted that due to the genetic distinctiveness of the two populations, translocations of individuals between them is not a viable option unless one population were to go extinct. Conservation effort should focus on the protection and expansion of each individual population and the patches of suitable habitat on which they rely. Ideally, rather than promoting the continued isolation of these populations, there is a need to restore connectivity

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and gene flow between the two populations through re-introductions at suitable intervening sites. Although both populations occur in a National Park, implementation of conservation practice for both populations needs to be of priority to ensure the adequate protection of this Peninsula endemic. Of particular concern is the Cape Point population, due to their low genetic diversity and the low number of adults, tadpoles and quantity of spawn. This population should be of local conservation priority and receive immediate and careful monitoring (see Appendix D for management recommendations).

The inability to locate historical populations reinforces the fact that the remaining populations on the Peninsula are of high conservation importance. In addition, if these are in fact the last two remaining extant breeding populations, should a catastrophic event occur (e.g. a change in seasonality and/or rainfall or a high intensity fire) there is a risk that one or both of these populations could disappear. Their specific habitat requirements, restricted dispersal abilities and contracted breeding times coupled with their low genetic diversity may hinder their ability to recover from such an event. Although it is not possible to unequivocally proclaim that the historical populations have been lost, it would be a misapprehension to ignore the possibility that Silvermine and Cape Point constitute the last remaining populations. Failure to respond to the findings provided here may lead to the demise of an evolutionary distinct lineage and result in yet another 'silent extinction'.

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Chapter 3

Study Review and Synthesis

CONCLUSIONS

In recent years, the utilisation of molecular techniques to infer genetic diversity and thus the long-term viability of wild populations and species has been central to conservation biology. However, its application is often overlooked when implementing practical local management plans as well as national and international policies (Laikre 2010). Unfortunately, this is possibly where it matters the most. Additionally, the application of conservation genetics has tended towards the study of the most tractable species (Beebee 2005), leaving those that are narrowly distributed, isolated and thus potentially more vulnerable by the wayside. Declines of amphibian populations and species represent a phenomenon that goes beyond the general biodiversity crisis, whereby declines are common in areas that are protected and/or relatively pristine. These types of 'enigmatic declines' are potentially the most difficult to redress as they reveal an inadequacy in the traditional management technique of habitat protection (Collins and Storfer 2003).

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Through the application of phylogenetic and population genetic analysis combined with the collation of historical distribution data, this study offers interesting insight and practical management options for the mitigation of the possible 'enigmatic decline' of a highly restricted and isolated species of toad. By resolving the taxonomic status of *Capensibufo rosei*, a species that was, until recently, thought to exist in eight general locations on and off the Cape Peninsula, and through assessing their vulnerability to extinction risk through population genetic methods, it has been possible to identify specific units for conservation priority. The evidence and management recommendations provided by this study, if acted

upon by national and international conservation bodies, may contribute to the future survival of what is now known to be a rare Peninsula endemic precariously close to extinction.

FUTURE RESEARCH

It is evident that complex phylogeographic relationships occur amongst populations of *Capensibufo* sp found in the fold mountains east of the Cape Peninsula. With further analysis of these populations it is possible that, like those populations found on the Peninsula, they too will exhibit strong population structure and divergence across small geographic ranges. This avenue of research is of conservation importance to ensure the protection of what may be discrete and evolutionarily distinct lineages. For the species east of the Cape Peninsula, further phylogenetic analysis and traditional forms of nomenclature will have to be applied to enable the species to be named. Additionally, although of less urgency, the use of nuclear markers may enable a more strict application of units for conservation for both species. For *C. rosei sensu stricto*, the use of additional markers may provide useful insights. For example, microsatellites can provide estimates of effective population size (N_e) from just a single generation, which may provide further evidence that these populations are small and highly vulnerable (Beebee and Griffiths 2005). In addition, microsatellites, being highly polymorphic, can also provide important information regarding population bottlenecks and thus changes in expected heterozygosity (Cornuet and Luikart 1996).

With monitoring being an important part of species conservation, there is also a need for further field-work to monitor extant breeding sites as well as those historical sites that have not been re-located. Observations are needed to provide further information on life history traits and dispersal abilities for this little known species. Aside from a handful of studies that briefly touch upon its systematics and biology (Grandison 1980; de Villiers 2004;

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Cunningham and Cherry 2004; Frost et al. 2006; Tolley et al. 2010) little has been published on *C. rosei*. In particular, studies of dispersal capabilities would provide useful information when trying to restore connectivity. Additionally, due to difficulties in finding the species outside of breeding season, virtually nothing is known about its habitat requirements during the rest of the year. Until this gap is redressed, it is impossible to conserve suitable habitat to support the species at all stages of its life cycle.

Although it is not possible to identify definitively a proximate cause for the apparent demise of *C. rosei* from 10 sites on the Cape Peninsula, it is possible to infer the circumstances at some sites. Numerous sightings in three areas of Table Mountain (Platteklip Gorge, Echo Valley, and Maclear's Beacon) suggest that there once were several breeding sites here. Regular searches in these areas during the breeding season over a number of recent years, have resulted in no observations (Dr. G.J. Measey. pers. comm.), despite the presence of suitable habitat. Populations at these sites, along with others found on the Peninsula, appear to have experienced some form of 'enigmatic decline'. Alternatively, it may be that a high level of human disturbance (especially at Platteklip Gorge, which sits along a main footpath) has caused their disappearance at these sites. With more than 780,000 visitors estimated to have visited Table Mountain in 2002 (SANParks 2004), it is difficult to imagine that there has not been an impact on the ecosystem. Additionally, during the 1850s Table Mountain and its surrounds were afforested with pine plantations (Huntley 1999), although the plantation never extended to the areas where this species was historically recorded (C. Cowel. pers. comm.). Most of the pines have since been cleared; a process which continues today. Pine plantations on Table Mountain have been associated with a reduction in native plant and invertebrate diversity and the lowering of the water table (Pryke and Samways 2009). A change in the hydrology on Table Mountain may have led to the loss of suitable habitat

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during the time of the plantations. Similarly, the breeding site that was recorded east of the Old Cape Road, may have suffered from changes in hydrology due to the construction of the road which began in 1965 and was completed in December 1968 (C. Atkins. pers. comm.). In this fire-driven biome, there is also a possibility that due to the increased fuel load of alien plants, or the lack of an appropriately managed fire regime, a fire of high intensity may have occurred, leading to the loss of individuals or appropriate habitat. Additional information on fire frequencies, habitat alteration from commercial timber plantations and their subsequent clearance, plus records of rainfall patterns could provide useful insights the factors leading to the demise of populations at these sites. Such information also could be useful to guide the future management of the remaining populations on and off the Peninsula.

COMPLICATIONS OF THE STUDY

Possibly the most problematic aspect of this study was the inability to confidently discern between an absence of occurrence and absence of observation, causing the inference of local extinctions to be somewhat circumspect. Although these types of data are important lines of evidence, observed absences are rarely recorded or drawn upon in the study of amphibians (Skelly et al. 2003), possibly due to the uncertain nature of the data. Additionally, when dealing with a species such as *C. rosei*, which is a small, rare and silent species, searching large areas of suitable habitat is extremely difficult with searches being directed and timed in such a way as to maximise the probability of detection. In a study conducted by Tanadini and Schmidt (2011), there was a direct correlation between population size and detection probability, with the likelihood of a population going undetected increasing with decreasing population size. This can consequently result in a misinterpretation of results and perhaps more worryingly, lead to the discontinuation of species-specific, or in this case population-

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specific, management actions. This compounds the need to continue monitoring breeding sites that seem to have disappeared.

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APPENDICES

Appendix A. Historical records of Rose's mountain toad, *Capensibufo rosei*, on the Cape Peninsula. Records have been consolidated by sighting. For the full data set, including grid references, altitudes, museum accession numbers, and additional field notes, please contact the author).

Year	Month	Date	Location	Collector	Record Type
Silvermine Nature Reserve					
1901	September	-	River valley	W.F. Purcell	Museum specimen
1925	-	-	Muizenberg	W. Rose	Museum specimen
1925	-	-	Muizenberg	Unknown	Museum specimen
1960's	-	-	Near waterfall	R.C. Boycott	Anecdotal
1971	August-October	-	Silvermine Plateau	R.C. Boycott	Field Notes
1971	August	-	East of Old Cape Road	R.C. Boycott	Field Notes
1971	August	-	Steenberg Plateau	W.R. Branch	Anecdotal
1972	August	13	Steenberg Plateau	R.C. Boycott	Museum specimen
1972	April	6	East of Old Cape Road	R.C. Boycott	Field Notes
1972	August	13	East of Old Cape Road	R.C. Boycott	Field Notes
1979	August	26	Steenberg Plateau	A. de Villiers	Museum specimen
1979	September	23	Steenberg Plateau	W.R. Branch	Museum specimen
1979	September	-	Steenberg Plateau	R.C. Boycott	Anecdotal
2008	August-September	-	Steenberg Plateau	K. Tolley/J. Measey	GPS locality/DNA sample
2009	August-September	-	Steenberg Plateau	K. Tolley/J. Measey	GPS locality/DNA sample
2010	July-September	-	Steenberg Plateau	K. Tolley/J. Measey	GPS locality/DNA sample
2011	July-October	-	Steenberg Plateau	This study	GPS locality/DNA sample
Cape of Good Hope Nature Reserve (Cape Point)					
1973	September	2	Olifantsbos	R.C. Boycott/B. Rose	Museum specimen
1988	July	8	Cape of Good Hope Nat. Res.	L.A.C. Hoffmann	Museum specimen
1996	-	-	Geps Dam	M. Picker	Field Notes
2010	-	-	Geps Dam	J. Measey	Field Notes
2010	September	2	Gilli Dam	J. Measey	Field Notes
2011	March	25	Geps Dam	J. Measey	Field Notes
2011	August-September	-	Gilli Dam	This study	GPS locality/DNA sample
Table Mountain area					
1927	August	21	Maclear's Beacon	W. Rose	Field Notes
1927	December	27	Table Mountain	K.H. Barnard	Museum specimen
1950	December	15	Western Table	Lund University	Field Notes
1950	December	15	Echo Valley	Lund University	Field Notes
1958	January	7	Table Mountain	Unknown	Museum specimen
1960	July	-	Near mountain club hut	C.E. Gow	Museum specimen
1960	July	-	Table Mountain	Unknown	Museum specimen
1960	July	-	Echo Valley	Unknown	Museum specimen
1961	January	11	Maclear's Beacon	J. Visser	Museum specimen
1965	January	2	Near mountain club hut	C.E. Gow	Museum specimen
1965	September	-	Top of Table Mountain	J. Visser	Museum specimen

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1966	December	8	Table Mountain	J. Visser	Museum specimen
1969	May	21	Table Mountain	P. van den Elzen	Museum specimen
1971	January	4	Table Mountain	J. Visser	Museum specimen
1973	September	27	Table Mountain	J. Visser	Museum specimen
1973	September	29	Maclear's Beacon	J. Visser	Field Notes
1973	November	4	Table Mountain	J. Visser	Museum specimen
1973	December	15	Maclear's Beacon	J. Visser	Field Notes
1973	-	-	Platteklip Gorge, Table Mountain	J. Visser	Anecdotal
1979	-	-	Table Mountain	Lund University	Museum specimen
1979	April	5	Top of Table Mountain	W.R. Branch	Museum specimen
1983	-	-	Table Mountain	J. Visser	Museum specimen
Other Sighting					
1904	January	-	Chapmans Peak	R.M. Lighfoot	Museum specimen
1907	May	26	Cape Peninsula	C. French	Museum specimen
1915	-	-	Kalk Bay	H. Power	Museum specimen
1925	February	-	Jackal's Drift	W. Rose	Field Notes
1925	February	-	Kalk Bay	W. Rose	Field Notes
1978	January	8	Ocean View, Kommetjie	A. de Villiers	Museum specimen
2004	August		Blackhill Road	K. Louw	Anecdotal
Negative Observations					
1974	September	22	Platteklip Gorge, Table Mountain	J. Visser	Anecdotal
1985	August/September	-	Maclear's Beacon	M. Cherry	Anecdotal
1985	August/September	-	Oliphantsbos, Cape Point	M. Cherry	Anecdotal
1998	-	-	Maclear's Beacon	M. Cunningham	Anecdotal
1998	-	-	Platteklip Gorge, Table Mountain	M. Cunningham	Anecdotal
2011	August/September	-	Platteklip Gorge, Table Mountain	This study	Field Notes
2011	August/September	-	Maclear's Beacon	This study	Field Notes
2011	August/September	-	Echo Valley	This study	Field Notes
2011	August/September	-	Near mountain club hut	This study	Field Notes
2011	August/September	-	Blackhill Road	This study	Field Notes
2011	August/September	-	Chapmans Peak	This study	Field Notes
2011	August/September	-	Oliphantsbos, Cape Point	This study	Field Notes
2011	August/September	-	Ocean View, Kommetjie	This study	Field Notes
2011	August/September	-	East of Old Cape Road	This study	Field Notes
2011	August/September	-	Geps Dam	This study	Field Notes
2011	August/September	-	Jackal's Drift	This study	Field Notes

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Appendix B. Results from analysis of molecular variance (AMOVA) preformed in Arlequin 3.1.1 (Schnider et al. 2007).

Conventional F-Statistic from haplotype frequencies (P<0.001***)

Source of Variation	d.f.	Sum of squares	Variance Components	Percentage of variation
Among Populations	1	12.512	0.40200 Va	79.77
Within Populations	60	6.117	0.10196 Vb	20.23
Total	61	18.629	0.50395	
Fixation Index			F_{ST}:	0.79770

Distance method: Tamura and Nei (P<0.001***)

Source of variation	d.f.	Sum of squares	Variance Components	Percentage of variation
Among populations	1	27.550	0.88898 Va	89.31
Within populations	60	6.384	0.10640 Vb	10.69
Total	61	33.934	0.50395	
Fixation Index			Φ_{ST}:	0.89311

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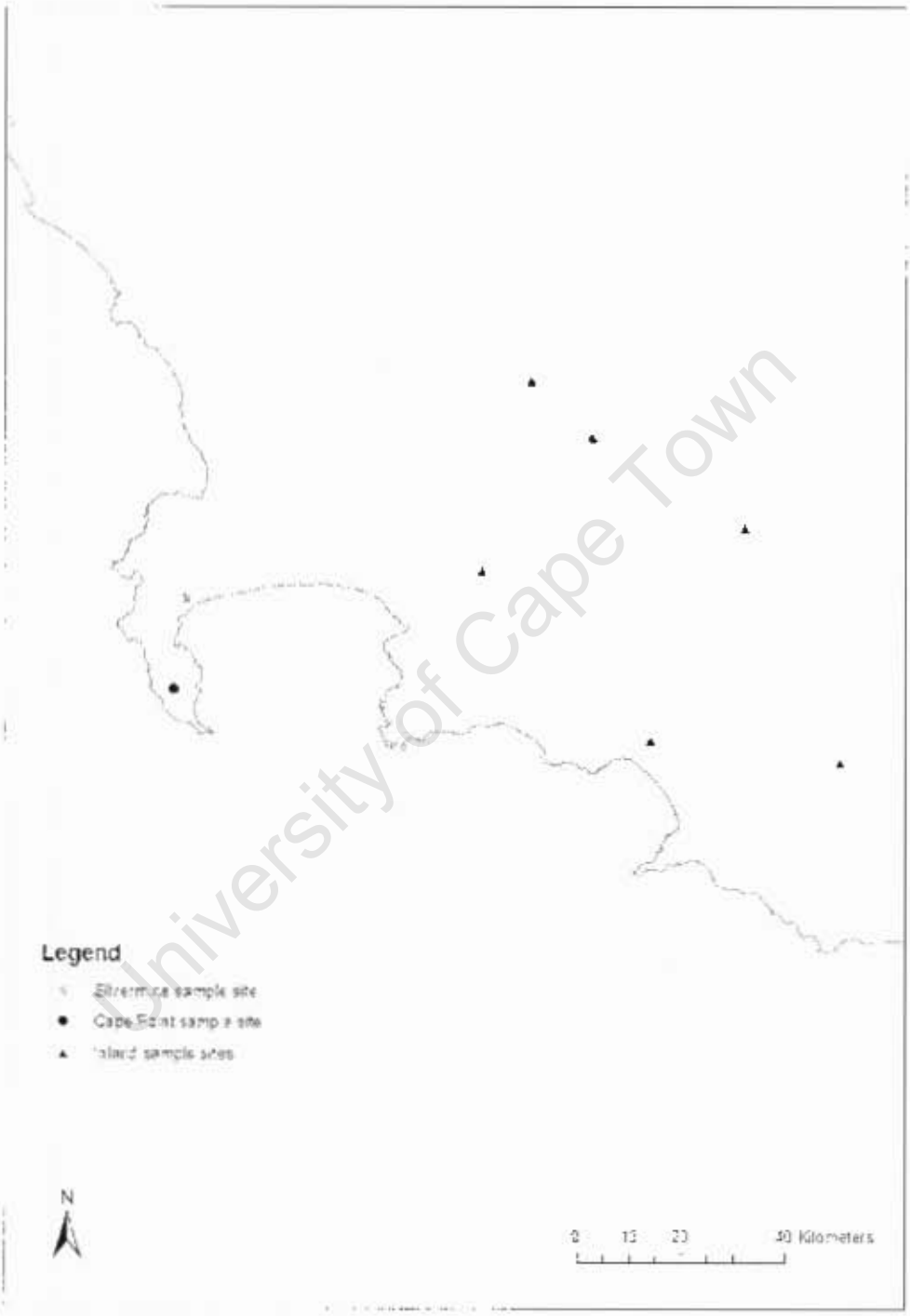
Appendix C. Localities of all known populations of *Capensibufo rosei* (data points provided by Dr. G. J. Measey).



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Appendix D. Management recommendations for *Capensibufo rosei* on the Peninsula, for submission to South African National Parks (SANParks). Completed by Dr. G.J. Measey and E. R. Cressey.

MANAGEMENT RECOMMENDATIONS

ANNUAL PROGRESS REPORTS – APPENDIX B

South African
NATIONAL PARKS

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Project title: Investigating the biodiversity, gene flow and dispersal capabilities of amphibians in Table Mountain National Park.

1. **Senior Researcher:** Dr. John Measey

   Applied Biodiversity Research

   Kirstenbosch Research Centre

   South African National Biodiversity Institute

   P/Bag X7, Claremont 7735

2. **Study sites:** Cape Peninsula

   a. **Silvermine East:** *Steenberg Plateau 34.10092S 18.44863E*

   b. **Cape of Good Hope:** *near Gilli Dam 34.30366S 18.43953E*

3. **Recommended action to be taken:**

a. Re-route head of footpath through breeding area in Steenberg Plateau.

Capensibufo rosei breed in shallow puddles of water that are associated with seepages. These puddles may be made through the action of hikers, but hiking during breeding times (August – November) could jeopardise breeding success in puddles that fall on the path. While the disturbance from small numbers of hikers would probably be negligible (or at least equivalent to natural large mammal movements), increasing numbers of walkers and dogs in the area elevates the risk to the point where a year's entire reproductive output might be lost.

In order to reduce the potential risk from hikers, we propose that the path through the site be moved to the south of this area, and that a board walk be used to channel hikers through this seepage area. This work should be done during the summer months (November to June) to prevent disturbance at the breeding site.

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b. Deepening puddles in breeding area Silvermine and Cape Point

After rains in June/July but before breeding (August/September) we propose to deepen some existing seeps away from the previous year's breeding puddles, but within the general area. It is hoped that deeper puddles would hold water for longer into the summer and result in enhanced breeding success.

c. Monitoring breeding success and time at Silvermine and Cape Point

Although very little is known about the breeding biology of this species, we know that reproductive output is quite high (30 to 50 eggs per female). Monitoring development of eggs and tadpoles can give information about comparative success (between sites and years).

We have a non-intervention method which uses a simple digital camera and laminated grid sheet to monitor tadpole growth. We would happily train and support park staff or honorary rangers to conduct this monitoring on a regular basis during the breeding period.

d. Location of additional breeding sites

Perhaps the most important goal is to locate more breeding sites of this species on the Cape Peninsula. We would welcome an opportunity to instruct park staff and honorary rangers in how to identify this species and its eggs and tadpoles. In particular, it would be useful to get feedback for areas where the animals are not found. All park staff and honorary rangers could help with the use of cyber-trackers and/or photos uploaded to iSpot of all amphibians on the peninsula.

e. Ex-situ breeding

We recommend that an ex-situ breeding programme be initiated for this species using a partner organisation through the African Association of Zoos and Aquariums.

f. Restore connectivity

It is known that the two breeding sites on the Peninsula are separate populations that display distinctiveness at a genetic level. This means that translocation of individuals among Silvermine and Cape Point is not a viable option, unless one population dies out. However, since it is clear that the species once existed in numerous locations on the Peninsula that would have facilitated gene flow between the two populations and thus reduce the deleterious effects associated with small, isolated populations such as these, conservation effort should focus on restoring connectivity between sites. This could occur through re-introductions at suitable sites, using animals from Silvermine for sites north of the Fish Hoek Valley (which

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forms a natural barrier dividing the northern and southern Peninsula mountain chain), and those from Cape Point for sites to the south of the valley.

4. **Motivation for action:**

Currently, these are the only two known breeding sites for this species. Although it is possible that more exist, old breeding sites have not been re-located over several decades. Each breeding site has the potential to seed much larger areas with this species. The suggested actions are designed to provide a greater understanding of this species and to allow for appropriate interventions should sites be threatened by (for example) alien vegetation.

Recent population genetic work on the species has revealed that they have extremely low genetic diversity, especially those found at the Cape of Good Hope Nature Reserve. The risk of extinction is higher for species that display such low levels of genetic diversity through an inability to adapt to environmental changes and survive random stochastic events.

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5. **Recommended methods to be used:**

- Re-routing foot path
- Digging puddles deeper – with a hoe by scraping down accumulated mud and dead vegetation
- Monitoring tadpoles – with a digital camera and laminated grid paper
- Finding new sites – Cyber-tracker and digital camera
- Extending suitable areas of habitat

6. **Recommended time frames:** *(actions a through e above)*

a. Present-2012 (end before May 2012)

b. June/July 2012 - onwards

c. Winter 2012 – onwards

d. Winter 2012 – onwards

e. Within the next 5 years

f. Winter 2012-onwards

7. **Possible unintended consequences and other potential negative impacts:**

a. Moving the path will cause less puddles within the breeding area which may negatively impact breeding sites. This will be mitigated by (b).

b. Deeper puddles may not be chosen by toads.

c. Amphibians are susceptible to disease, and dedicated equipment (e.g. laminated sheets for photography) should be used at each site, and not at both sites. Staff and volunteers must take adequate measures to prevent spread of disease.

8. **Other comments:**

Capensibufo rosei from the Cape Peninsula was found to be a distinct species from those in the adjacent Cape Fold Mountains (Tolley et al. 2010). The sites found on the Peninsula form a single genetic lineage, but these sites are distinct at a population level. This indicates that those populations found on the Peninsula require a level of separate management, with the Cape Point population (due to their low genetic diversity) being of priority. IUCN re-assessment of the *C. rosei* is an ongoing process; this evidence should be used to change the status of this newly recognised Peninsula endemic from Vulnerable to Endangered or even Critically Endangered.

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