

# Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.

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## Abstract

Taxa with different degrees of habitat specificity have been known to be susceptible to varying factors imparting fragmentation within forests. Therefore, delimiting their evolutionary history through population genetics is bound to shed light on the impact paleoclimatic and biogeographical events may have had in shaping their contemporary genetic structure. This study aimed at examining evolutionary relationships among populations of three anuran taxa; *Anhydrophryne rattrayi*, *Arthroleptis wahlbergii* and *Cacosternum nanum* within two indigenous forest types, the Afromontane forests and Indian Ocean coastal belt forests (IOCB) in South Africa. The former two species are leaf litter forest dependent and direct developing frog species whereas the former is a generalist species dependent on open water throughout its life stages. Phylogenetic reconstructions were inferred from combined mitochondrial DNA sequence data (16S rRNA and Cytochrome *b*) whereas only Cytochrome *b* data was analysed for phylogeographic analyses. Analyses of phylogenetic relationships within the two forest specialists (*Anhydrophryne rattrayi* and *Arthroleptis wahlbergii*) detected strongly supported clades with marked genetic variation and structure between populations, absence of shared maternal haplotypes indicating limited maternal gene flow between populations of these species. Lineage diversification within forest dwelling species followed the Plio-Pleistocene climatic perturbations indicating the influence of these paleoclimatic events as well as barriers in isolating populations to several refugia habitats. Contrarily, the generalist species, *Cacosternum nanum* revealed presence of low support and unresolved phylogenetic structure, connectivity between populations indicated by high maternal gene flow and the recovery of younger lineages suggesting that the species' ecology may aid its dispersal abilities, subsequently, increasing its persistence, even during climatic stresses. Coupled with the different ecological mechanisms and life history traits of these taxa, populations may potentially be reproductively isolated and overtime, result in cladogenesis. This study thus suggests conservation management and decisions to be cognisant of the genetic uniqueness recovered using mtDNA of the forest specialist populations given the already fragmented habitats they occur in. Overall, this study lays grounds for several interesting biological questions such as possible taxonomical reclassification of the *A. wahlbergii* Mbotyi population and other statistical inferences made such as the inclusion of nuclear loci in the dataset.

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# **CHAPTER ONE INTRODUCTION**

## Chapter 1

### Introduction

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#### 1. Introduction

South Africa is a biodiversity rich country (Ricklefs, 1987; Lawes, 1990; Lawes *et al.*, 2000; Algotsson, 2009) with several biomes, each representing a characteristic climate. The forest biome in South Africa is one of the most poorly studied biomes in the country (Mucina and Geldenhuys, 2006; Mucina and Rutherford, 2006). This biome is also one of the smallest, covering <0.5% of the total land surface of a fragmented nature (Low and Rebelo, 1996; Bond, 2008). Although the floristic diversity and endemism in forests are well documented, studies on vertebrate and invertebrate endemism in the region are greatly understudied (Van Wyk and Smith, 2001; Pimm, 2007; Perera *et al.*, 2011). With forests known to be partly driven by their fire suppressing abilities (Bond and Zaloumis, 2016), it is not surprising that in South Africa, forests occur in high rainfall areas (Eeley *et al.*, 2001; Mucina and Rutherford, 2006) which are scattered along the coastline, from south west of the Western Cape Province towards the Eastern Cape Province reaching the KwaZulu-Natal Province (KZN) border with Mozambique with some isolated patches of Afromontane forest in Mpumalanga Province.

The Eastern Cape Province has the two main indigenous forest types in Africa; the Afromontane and Indian Ocean Coastal Belt (IOCB) forests (coastal forests hereafter) (White, 1981; Timberlake and Shaw, 1994; Von Maltitz *et al.*, 2003; Mucina and Rutherford, 2006). Strong congruence patterns exist between the biomes' biodiversity (Mucina and Rutherford, 2006) and paleogeographic links with other African indigenous forests (White, 1983; Timberlake and Shaw, 1994; Mucina and Rutherford, 2006). It is therefore important to examine the evolutionary history of taxa with different degrees of habitat specificity in these forests as they tend to be exposed to varying factors impacting fragmentation within these habitats. Forest dependent species such as those exhibiting limited mobility due to life history traits are anticipated to retain strong genetic signatures of historic biogeographical events. Thus, in a changing climate and environment, such studies can ultimately be used in the conservation management of taxa.

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## 1.1 Afromontane forests

Afromontane forests occur as fragmented patches concentrated in high altitude areas in South Africa, northern Zimbabwe, Malawi, northern Ethiopia, the Eastern Arc Mountains of Kenya and Tanzania, western Cameroon as well as some parts of Angola (White, 1983; Timberlake and Shaw, 1994; Mucina and Rutherford, 2006). The nature and history of these forests is described variously by several authors. For example, they are thought to be relicts; representing an ancient and persistent habitat (Acocks, 1953; Lawes, 1990; Scott *et al.*, 1997; Eeley *et al.*, 1999; Lawes *et al.*, 2000; Wethered and Lawes, 2003) widely distributed during mesic periods of the Oligocene and Miocene, periods in which mountain ranges were formed, forests contracted and grasslands expanded (White, 1978; Cooper, 1985; Lawes, 1990; Daniels *et al.*, 2016). Some authors describe Afromontane forests as representing recent invasions of forests (Cowling, 1983; Meadows and Linder, 1989) whereas some argue that they have always been fragmented (Midgley *et al.*, 1997). Though the origin of these forests may be considered complex (White, 1981) the influence paleoclimatic events had on the structure and distribution of forest biota is not debatable (Avisé and Walker, 1998; Hewitt, 2004; Provan and Bennett, 2008).

Paleoclimatic events and fluctuations are thought to have influenced species distribution and composition through time (Tolley *et al.*, 2006; Araújo and Al, 2008; Jackson *et al.*, 2009; Blach-Overgaard *et al.*, 2013; Svenning *et al.*, 2015). The Oligocene and Miocene represented periods of uplift in southern Africa (Bauer, 2010). These periods marked the onset of orogenic activity (Sepulchre *et al.*, 2006; Partridge *et al.*, 2010; Kaufmann and Romanov, 2012) in the region resulting in the mountain ranges seen today. Coupled with the warm Agulhas current, these mountain ranges created a rain shadow effect that has kept forests on windward slopes moist in this region (Neumann and Bamford, 2015) whereas the rest of the sub-continent was intensely dry with the development of the proto-Benguela current in the Late Miocene (Siesser, 1980; Sepulchre *et al.*, 2006). A mid-latitude glaciation occurred in the Late Pliocene, with the formation of the Arctic Ice cap (Knies *et al.*, 2014). Climate during this time fluctuated between glacial and interglacial periods (Deacon, 1983; Tyson, 1986; Deacon and Lancaster, 1988; Cutler *et al.*, 2003). Southern Africa however, did not undergo the dramatic glaciation brought about during these periods, but the effects were felt across the continent (Dolman and Joseph, 2012). The mesic-xeric cycles prevalent during the Pleistocene resulted in vegetation changes from



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closed wet forests to open dry savannas (Tolley *et al.*, 2008; Neumann and Bamford, 2015), subsequently reducing expanses of forests to patches, decreased faunal population sizes and impelled shifts in range distributions (Hewitt, 2000; Davis and Shaw, 2001; Hewitt, 2004; Hill *et al.*, 2011; Dolman and Joseph, 2012).

The advent of the Last Glacial Maximum (LGM) about 18 000 years ago, characterized by dry and cool temperatures (Geldenhuys, 1989; Scott *et al.*, 1997) encouraged grassland expansion, further reducing forests to fragments, confining some species to refugia (Hamilton and Taylor, 1991; Fjeldså and Lovett, 1997; McDonald and Daniels, 2012). However, a wet climate subsequently followed, assisting forests to re-establish and expand (Hamilton, 1981). Thus, the LGM presented varying effects on the distribution of flora and fauna in Africa (White, 1983; Meadows, 2001). For example, in South Africa, the Eastern Cape forests, KwaZulu-Natal scarp forests and the eastern Mpumalanga forests are known to have underwent considerable contractions (Lawes, 1990) whilst some forested areas around the Lake Malawi catchment area expanded (DeBusk, 1998; Irving, 1998). Limited quantity of pollen collected and analysed from clay deposits in the Afromontane forests of South Africa and Zimbabwe indicated that grasslands dominated the ecosystem before the Holocene, 11 700 years before present suggesting that forests were fragmented before the LGM (Meadows and Linder, 1989; Meadows and Linder, 1993). In contrast, greater pollen quantities found around the Lake Malawi catchment area suggests forests were more continuous and widespread during the LGM, fragmentation only occurring with the coming of the Holocene (DeBusk, 1998).

In South Africa, Afromontane forests occur in allopatric fragments along the mountainous interior where moisture is aseasonal (Low and Rebelo, 1996). They occur from southwest of the Western Cape Province to the northeast of the Limpopo Province (Hobday, 1976). Currently, Afromontane forests are discontinuous and restricted to high altitude, cool and moist areas, separated by expanses of dry corridors that likely limit the dispersal capability of small, less mobile, forest dependent taxa.

## **1.2 Coastal forests**

Several views on the formation of coastal forests have been presented. Sepulchre *et al.* (2006) asserts the uplift of the southern African subcontinent at the beginning of the Pliocene to coastal

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forest establishment, as formerly submerged areas were exposed to becoming dryland. In addition, the establishment of modern sea levels and the stabilization of the formerly submerged areas encouraged coastal forest expansion from the Mozambique coast into KZN Province and parts of the Eastern Cape (Lawes, 1990; Eeley *et al.*, 1999; Botha *et al.*, 2003). Thus, coastal forests represent a fairly recent habitat (Van Wyk, 1994; Lawes *et al.*, 2007). Another view is that coastal forests are the fragmented remnants of a continuous and persistent Pan African forest block that existed between the Eocene and Miocene (Burgess *et al.*, 1998). The desiccation in climate, coupled with geological events such as the uplift of East Africa further reduced forest cover, separating coastal forest into western and eastern patches. With climatic cycles shifting between wet and dry, faunal exchange between forest patches may have been possible, enhancing faunal and floral links with other forest types. Thus, coastal forests remarkably harbour a high degree of endemic flora and fauna (Lawes, 1990; Van Wyk and Smith, 2001; Perera *et al.*, 2011). The extent of the coastal forests in South Africa coincides with two centres of endemism (Mucina and Rutherford, 2006) which can be attributed to the mixing of forest elements that took place during the global cooling and aridification events of the Late Miocene and Pliocene which altered species distributions (Sepulchre *et al.*, 2006), restricting some taxa to refugia. Similar to the Afromontane forests, coastal forests underwent unrestrained climatic changes induced by the decrease in temperature during the LGM, reduced precipitation (Botha *et al.*, 1992), weak Agulhas current (Prell *et al.*, 1980) and low sea surface temperatures (Van Zinderen Bakker, 1982). However, the climate ameliorated rather rapidly after the climatic extinction filtering event of the LGM and favorable mesic conditions re-established the biotic community in these coastal forests (Tinley, 1985; Tyson, 1986). In addition, the dispersal and recolonization of forest fauna post LGM from tropical East African refugia into KZN scarp forest refugia and expansion of the coastal forests into scarp forest of the KZN and the Eastern Cape Province (Lawes, 1990; Lawes *et al.*, 2007) accounts to the high species richness within coastal forests. Scarp forests are the contemporary connection between the Afromontane forests and the coastal forests and overall, represent refugia for Afromontane fauna during climatic alterations of the ice age (LGM) (Lawes *et al.*, 2007).

Coastal forests in South Africa represent the most threatened forest type (Driver *et al.*, 2012), occurring along a 800km coastal narrow strip between the Eastern Cape Province and the KZN Province (Moll and White, 1978; Mucina and Rutherford, 2006). In South Africa as well as in

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Mozambique, Tanzania, Kenya and the southernmost part of Somalia, coastal forests generally occur on marginally flat terrain or in deeply incised valleys which can potentially act as physical barriers and isolate faunal species populations (Moll and White, 1978; Mucina and Rutherford, 2006).

The paleoclimatic events that spanned over millennia shaped and influenced the current distribution of flora and fauna (Araújo and Al, 2008; Jackson *et al.*, 2009; Blach-Overgaard *et al.*, 2013; Svenning *et al.*, 2015). Potentially, these shifts impacted the phylogeographic structure of forest dependent taxa (Schneider *et al.*, 1998; Willis and Whittaker, 2002; Austin *et al.*, 2004; Hoffman and Blouin, 2004; De Queiroz, 2007b; Edwards *et al.*, 2007) resulting in allopatric speciation, cladogenesis and extinction in some taxa (White, 1981; Daniels *et al.*, 2016). Therefore, taxa living in these two forest habitats (Afromontane forests and the coastal forests) should exhibit the impact of both ancient and recent climatic shifts; particularly for taxa that are highly sensitive to moisture availability and limited mobility such as for example, amphibians.

### **1.3 The biology of amphibians: Anura**

Amphibians represent one of the most diverse radiations of tetrapods (Vences *et al.*, 2003; Measey *et al.*, 2007). Globally, amphibian species richness is estimated at around 7874 species, including 6945 anurans, 721 newts and 208 caecilians (Frost, 2018). Anura, the most widely distributed order of amphibians currently stands at 132 described species in South Africa (AmphibiaWeb, 2018), 25 of which are of conservation concern (IUCN, 2018). Amphibians are facing alarming biodiversity losses (Stuart *et al.*, 2004; Fouquet *et al.*, 2010). Population declines exacerbated by sensitivity to environmental change and human mediated changes have earned amphibians recognition, especially anurans given they are suitable indicators, commonly used in assessing global ecological status (Wake and Vredenburg, 2008; Murray *et al.*, 2011; Wake, 2012).

Anuran persistence and viability is important in countries such as South Africa where species of conservation concern are concentrated in centres of endemism (Minter *et al.*, 2004), which apart from facing further fragmentation from climate change, anthropogenic activities such as proposed developments and a growing human population also potentially threaten to alter species persistence in such areas (Castley and Kerley, 1996; Von Maltitz *et al.*, 2003; Berliner, 2005).

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Thus, knowledge of historical events and how they influenced current species ranges coupled with phylogenetics can aid in informed conservation and management efforts of both flora and fauna in the face of future climate and infrastructural projections.

The phylogeographic patterning of many Anuran species is closely linked to mesic and xeric climatic cycles (Vences and Wake, 2007). The latter observation has been confirmed by several phylogeographic studies on anurans (Mulcahy and Mendelson III, 2000; Harris, 2001; Macey *et al.*, 2001; Austin *et al.*, 2004; Lee-Yaw *et al.*, 2009). Anuran life history traits such as reduction in size, permeable skin (Gonzales-Voyer *et al.*, 2011; Pabijan *et al.*, 2012) make them useful organisms to test the impact of historical and biogeographical events (Beebee, 1977; Du Preez and Carruthers, 2009) as they promote speciation through isolation by distance and genetic differentiation (Carnaval *et al.*, 2009; Wollenberg *et al.*, 2011; Mynhardt *et al.*, 2015). Species characterized by limited dispersal capabilities, such as anurans (Beebee, 2005; Smith and Green, 2005) are exposed to climatic extinction filtering, and represent good non-model organisms used to assess the impact of climatic ameliorations (Duellman and Trueb, 1986; Blaustein *et al.*, 1994; Beebee, 1995; Lawes *et al.*, 2000; Gibbs and Breisch, 2001; Blaustein and Kiesecker, 2002; Blaustein *et al.*, 2003; Parmesan and Yohe, 2003; Lawes *et al.*, 2007; Lawler *et al.*, 2010).

Physiologically, anurans experience rapid dehydration, have a low tolerance to elevated temperature ranges, rendering them valuable for phylogeographic studies (Blaustein *et al.*, 1994). With anurans exhibiting strong geographic and reproductive site fidelity (Wake, 1991; Blaustein *et al.*, 1994; Blackburn, 2008b; Hutter *et al.*, 2013), habitat fragmentation can potentially isolate populations resulting in either cladogenesis or the extinction of species (Marsh and Pearman, 1997). Over short geographical distances, the genetic structure of anuran populations tends to be higher in comparison to more mobile animals allowing them to retain a strong signature of historical events that generated their contemporary population genetic structure (Zeisset and Beebee, 2008). Thus, the degree of genetic diversity in anurans is significantly correlated to habitat (Nevo and Beiles, 1991). The altering of the biotic landscape in the South African forests should have resulted in diverse phylogeographic patterning of anurans living in these habitats, with forest dependent species displaying the most marked population genetic structure, whereas species secondarily found in these habitats revealing the least amount of genetic differentiation (Blaustein *et al.*, 1994).

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Historically, the classification of anurans was based on morphological characters (Schjøtz, 1963; Poynton, 2003a; Blackburn, 2008a; Blackburn, 2009; Rödel *et al.*, 2009). However, the morphological characters used in the alpha taxonomy of the group are frequently conserved or convergent leading to an underestimation of its diversity within groups (Bickford *et al.*, 2007; Elmer *et al.*, 2007; Fouquet *et al.*, 2007a; Fouquet *et al.*, 2007b; Willis, 2017).

The advent and application of molecular systematics has significantly aided the delineation of species and recovered several novel lineages (Fouquet *et al.*, 2007a; Fouquet *et al.*, 2007b; Meir, 2008; Vieites *et al.*, 2009; Oliver and Lee, 2010; Conradie, 2014; Oliver *et al.*, 2014). Molecular phylogenetics has revealed historical patterns of range contractions and expansions, complex genetic connectivity, zones of genetic overlap, several cryptic lineages, new species descriptions and phylogeographic breaks among other discoveries in Anura that have aided the conservation management of several species (Shaffer *et al.*; Schneider *et al.*, 1998; Noonan and Gaucher, 2006; Vieites *et al.*, 2006; Channing *et al.*, 2013a; Channing *et al.*, 2013b; Conradie, 2014; Frost, 2016; Frost, 2018).

## 1.4 Study species

The Pyxicephalidae and Arthroleptidae are among the most interesting families of Anura in providing insights into biogeographic patterns in the Afrotropical region (van der Meijden *et al.*, 2005; Bittencourt-Silva *et al.*, 2016). Pyxicephalidae, diversified between the Late Cretaceous to Early Palaeocene, 69.9 million years ago (mya) (van der Meijden *et al.*, 2005; Bossuyt *et al.*, 2006; Roelants *et al.*, 2007; Wiens *et al.*, 2009; van der Meijden *et al.*, 2011). In Arthroleptidae, diversification occurred during the Late Cretaceous, 69.1 mya (Bossuyt and Roelants, 2009; AmphibiaWeb, 2018). The present study focused on three anuran species; *Anhydrophryne rattrayi* and *Cacosternum nanum* (family Pyxicephalidae) and *Arthroleptis wahlbergii* (family Arthroleptidae) in the fragmented Eastern Cape Province forests, South Africa.

### 1.4.1 *Anhydrophryne rattrayi* Hewitt (1919)

Previously a monotypic genus (Poynton, 1964; Burger, 2004), *Anhydrophryne* currently consists of three species *A. hewitti*, *A. ngongoniensis* (formerly considered *Arthroleptella*) and *A. rattrayi* (Dawood and Stam, 2006). *Anhydrophryne rattrayi* is a terrestrial leaf litter forest dependent

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frog, independent on open water in all its life stages (Poynton, 1964; Passmore and Carruthers, 1995; Burger, 2004; Dawood and Stam, 2006). Adults are known to be found near waterfalls whilst eggs are laid in moist leaf litter in forest ecotones, becoming tiny froglets during metamorphosis (Wager, 1986; Lambiris, 1988; Passmore and Carruthers, 1995; Channing, 2001; Burger, 2004; Dawood and Stam, 2006; van der Meijden *et al.*, 2011).

*Anhydrophryne rattrayi* is endemic to the Eastern Cape Province where its distribution is centralized in the Amatole Mountains (Channing, 2001; Burger, 2004; Minter *et al.*, 2004; Dawood and Stam, 2006). Species distribution data on *A. rattrayi* can be considered insufficient as there are few post-1996 records (Burger, 2004) and only a limited number of studies focusing on it. The conservation status of this frog has been reclassified several times, owing to its limited distribution, density and occurrence in severely fragmented and high elevation forested areas in the Amatole region of the Eastern Cape Province. Lambiris (1988) classified it as Restricted, with IUCN reassessments as Rare (R) in 1994, Low risk/near threatened (LR/NT) in 1996, Endangered (EN) in 2004. *Anhydrophryne rattrayi* is currently listed as Vulnerable (VU) (IUCN SSC Amphibian Specialist Group & South African Frog Re-Assessment Group, 2016) as awareness and studies on the species are increasing.

#### **1.4.2 *Arthroleptis wahlbergii* Smith (1849)**

Two species from the genus *Arthroleptis*, *Arthroleptis stenodactylus* and *Arthroleptis wahlbergii* occur in South Africa, (Channing, 2004). The genus is well known for taxonomic anomalies (e.g. Poynton and Loader, 2008) and with several newly described species, (Poynton, 2003b; Poynton and Loader, 2008; Blackburn *et al.*, 2009b; Blackburn *et al.*, 2010), cryptic lineages may be present within the Eastern Cape Province population. This study selected only the Eastern Cape Province populations of *Arthroleptis wahlbergii* (a more pronounced population is known from the KwaZulu-Natal Province forests). Current work in progress suggested that these two populations may represent separate species (Tolley *et al.*, 2018). *Arthroleptis wahlbergii* is typically associated with leaf litter and known to persist in indigenous forests, although it can be found in thickets and grasslands (Channing, 2004). Its reproduction is by direct development from eggs laid in clutches underneath damp leaf litter into a fully developed froglet (Channing, 2004).

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*Arthroleptis wahlbergii* is endemic to the east coast and adjacent interior of South Africa (Channing, 2001; Channing, 2004; Minter *et al.*, 2004; Du Preez and Carruthers, 2009). The IUCN conservation status of *A. wahlbergii* is currently of least concern (LC) (IUCN, 2016) owing to its abundance in its habitat. However, when the Eastern Cape Province coastal forest clade is separated as a distinct species, a conservation status reassessment will be warranted. Channing (2004) acknowledges a need for more substantial distributional information to evaluate its local conservation status as its habitat is severely threatened by infrastructural developments and clearing for agriculture.

### **1.4.3 *Cacosternum nanum* Boulenger 1887**

In *Cacosternum* 16 described species are presently known (Frost, 2018). The species are morphologically similar (Channing *et al.*, 2013b) and several new cryptic species have recently been discovered (Channing *et al.*, 2013b; Conradie, 2014). *Cacosternum nanum* previously contained two subspecies: *C. n. nanum* and *C. n. parvum* before the latter was formally elevated to species status (Channing *et al.*, 2013b). *Cacosternum nanum* is a widespread generalist species (Channing *et al.*, 2013b), occurring in various habitats including forests, fynbos, savanna thickets and grasslands (Scott, 2004). *Cacosternum* species are adapted to breeding in shallow temporary pools with small pigmented eggs laid in clutches anchored on the substrate (Scott, 2004). *Cacosternum nanum* is known to have the quickest metamorphic growth rate known in any frog; froglets leave the water 17 days after hatching (Duellman and Trueb, 1986).

The range of *Cacosternum nanum* in South Africa is confined to areas below the Drakensberg escarpment (Scott, 2004) which forms a barrier in the northwest from which it follows the relatively moist southern side of the Cape Fold Mountains and extends further inland until it reaches southern KwaZulu-Natal (Scott, 2004). A second but disjunct population is found north-east of KwaZulu-Natal Province, extending into adjacent areas of southern Mozambique (Scott, 2004). The widespread distribution of this species could be attributed to its ability to survive outside forested environments (Blackburn and Measey, 2009). The IUCN conservation status of *C. nanum* is of Least Concern (LC).

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## 1.5 Significance of study

Knowledge of evolutionary relationships between and within species is essential in the interpretation of biological variation (Hillis, 1991). Comparative phylogeography involves the reconstruction of variations in codistributed species (Hickerson *et al.*, 2009; Prates *et al.*, 2016). These variations in genetic patterning among species and within populations are attributed to features of their habitat and evolutionary history (Lewontin, 1974; Evans *et al.*, 1997; Leffler *et al.*, 2012). Studies in the fragmented southern Cape forests of South Africa have indicated that changes in species composition may have occurred because of forest contraction and expansion due to climate and landscape changes (Geldenhuys, 1997). Present patterns of species composition in different forests suggest that their high degree of similarity may have been established before major fragmentation of the forests in the Late Miocene and presence of geographical barriers such as mountain ranges, rivers or incised valleys (Geldenhuys, 1989; Lawes, 1990; Geldenhuys, 1997).

Using molecular data for *Anhydrophryne rattrayi*, *Arthroleptis wahlbergii* and *Cacosternum nanum* in the Eastern Cape forests, the current study applies a comparative phylogeographic approach aimed at examining whether present day patterns of genetic diversity in anurans reflect congruent, multi-taxa responses to the historical paleoclimatic events documented across Africa, especially those in southern Africa.

## 1.6 Study aim

The study aimed at examining the evolutionary relationships among populations of the three anuran taxa, *Anhydrophryne rattrayi*, *Arthroleptis wahlbergii* and *Cacosternum nanum* that have different habitat preferences and reproductive traits within Afromontane and coastal forests of the Eastern Cape Province, South Africa using mitochondrial and nuclear DNA sequence data (mtDNA and nuDNA).



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## 1.7. Study objectives and hypothesis

### 1.7.1 Objectives

1. To examine the evolutionary relationships among populations of the three anuran taxa, *Anhydrophryne rattrayi*, *Arthroleptis wahlbergii* and *Cacosternum nanum* within different forest subtypes in the Eastern Cape Province, South Africa.
2. To compare the phylogeographic structure among and within the three anuran taxa populations based on their habitat specificity.
3. To estimate the timing of divergences among and within populations of the three anuran taxa.

### 1.7.2 Hypotheses (H<sub>1</sub>)

#### **Hypothesis 1:**

*Anhydrophryne rattrayi* should exhibit marked population genetic structure due to its habitat specialization and endemism to the Amatole Mountains whereas *Arthroleptis wahlbergii* should exhibit intermediate genetic structure based on its ability to survive outside forest environments and *Cacosternum nanum* should exhibit less pronounced genetic differentiation due to its ability to survive in any habitat and reliance on water for survival and reproduction.

#### **Hypothesis 2:**

Cryptic lineages may be present within the two forest specialists, *Anhydrophryne rattrayi*, more so in *Arthroleptis wahlbergii* based on the morphological complexity of the genus *Arthroleptis* and their endemism in isolated forest habitats compared to the generalist, *Cacosternum nanum*, which occurs across a variety of habitats.

#### **Hypothesis 3:**

There would be marked divergence time differences between the three species as they currently exhibit noticeable variations in size, reproductive modes and habitat preferences indicating different evolutionary trajectories, with *Cacosternum nanum* expected to show the most recent diversification.

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# **CHAPTER TWO**

# **MATERIALS AND METHODS**

## Chapter two

### Materials and methods

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## 2. Materials and Methods

### 2.1 Sampling

A combination of visual encounter survey methods (hand capture) and standard drift fences with pitfalls (each trap array consisting of 3 x 10 m long and 50 cm high fences positioned in a Y-shape with four pitfall traps at the ends and middle) were used. Diurnal searches were conducted by actively looking for specific microhabitats such as leaf litter, dead tree logs, in and around water bodies and listening for distinct frog calls. Nocturnal searches were carried out with the use of headlamps or flashlights. The vulnerable species (*Anhydrophryne rattrayi*) was photographed, toe clipped and released back at the original capture site (except for up to five selected voucher specimens per site, where possible). Minimum handling time was applied, especially to those frogs that were toe clipped and all efforts were made to minimize suffering. Ethical clearance was obtained by Mr Werner Conradie, Curator of Herpetology, Port Elizabeth Museum (PEM), Port Elizabeth, South Africa who was present during all field work (Ethics number 2013-01 & 2017-02). A maximum of ten specimens per site, where possible were collected as vouchers for the IUCN listed least concern (LC) *Arthroleptis wahlbergii* and *Cacosternum nanum*. Animals were euthanized in a solution of tricaine methane sulfonate (M222) and water. Liver tissue was taken from vouchered *Arthroleptis wahlbergii* and *Cacosternum nanum* specimens, while sampling size were supplemented with toe clips of additional specimens which were released at capture site. A total of 206 liver and toe clip tissue samples were collected (Table 1). Forty-six samples of *Anhydrophryne rattrayi* were collected from five localities within Afromontane forests of the Amatole Mountains (Table 1; Fig. 1), a total of 46 samples of *Arthroleptis wahlbergii*; 38 from four Eastern Cape Province forests, eight samples from three KZN Province forests (Table 1; Fig. 2) and 112 samples of *Cacosternum nanum* were collected from 27 locations (Table 1; Fig. 3) within the Eastern Cape Province forests and two were collected from Nature's Valley, Western Cape Province. To illustrate the differences between *A. wahlbergii* populations from the Eastern Cape Province forests (southern lineage) and the KZN Province forests (northern lineage) as suggested by (Tolley *et al.*, 2018), a total of eight specimens was collected from the KZN Province forests. A single sample was

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collected from Pinetown, three from Krantzkloof Nature Reserve and four from Nkandla Nature Reserve, KZN Province. All tissue samples were stored in 100% ethanol until required for use in DNA work. All voucher specimens were fixed in 4% formalin for 48 hours and subsequently transferred to 50% isopropanol for long-term storage in herpetological collections of the Port Elizabeth Museum (PEM), South Africa (Appendix 1).

## 2.2 Outgroup selection

Outgroups selected were either sister taxa, the most recent common ancestor (MRCA). However, given that sister taxa for *Cacosternum nanum* is still not clear, two species within the genus were selected at random. For *Cacosternum nanum*, *C. boettgeri* and *C. capense* were used to root trees (Channing *et al.*, 2013b). *Arthroleptis stenodactylus* and *A. francei* were used to root for *A. wahlbergii* (Channing, 2001) and for *Anhydrophryne rattrayi*, the two sister taxa, *A. ngongoniensis* and *A. hewitti* were used (Dawood and Stam, 2006), where for the former, available sequences from GenBank were used while a sample received from the South African Biodiversity Institute (SANBI) was used for the latter.

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**Table 1:** List of the localities sampled for the three focal anuran species (*Anhydrophryne rattrayi*, *Arthroleptis wahlbergii* and *Cacosternum nanum*) and sequences generated per gene region selected during the present study. The abbreviation N.R is for Nature Reserve. N corresponds to the localities on the maps (Figs.1, 2 and 3). Numbers 38 to 40 represents *A. wahlbergii* samples from KZN not included on Figure 2. Letters A, B and C represent forest types with A=Afromontane, B=scarp and C=Coastal forest.

N	Locality	Forest Type		GPS Coordinates		Species	Sequences generated per loci			
		A	Province	Latitude	Longitude		16S rRNA	Cyt b	RAG1	Rhodopsin
1	Hogsback	A	Eastern Cape	32.606200°	26.961930°	<i>A. rattrayi</i>	7	7	1	1
2	Isidenge	A	Eastern Cape	32.687855°	27.278564°	<i>A. rattrayi</i>	11	11	1	1
3	Katberg	A	Eastern Cape	32.473600°	26.672510°	<i>A. rattrayi</i>	12	12	1	1
4	Kologha	A	Eastern Cape	32.532510°	27.363641°	<i>A. rattrayi</i>	13	13	1	1
5	Kubusi	A	Eastern Cape	32.558684°	27.315337°	<i>A. rattrayi</i>	3	3	1	1
6	Goso	B	Eastern Cape	31.433700°	29.633680°	<i>A. wahlbergii</i>	14	14	1	1
7	Hluleka N.R	C	Eastern Cape	31.818100°	29.300170°	<i>A. wahlbergii</i>	7	7	1	1
8	Mbotyi	C	Eastern Cape	31.428000°	29.726150°	<i>A. wahlbergii</i>	10	10	1	0
9	Silaka N.R	C	Eastern Cape	31.651000°	29.511920°	<i>A. wahlbergii</i>	7	7	1	1
10	Amatole forest	A	Eastern Cape	32.560305°	26.913851°	<i>C. nanum</i>	2	2	1	1
11	Baziya	C	Eastern Cape	31.580656°	28.391107°	<i>C. nanum</i>	6	6	1	1
12	Dwesa N.R	C	Eastern Cape	32.287920°	28.867940°	<i>C. nanum</i>	12	12	1	1
13	Eels Cave	B	Eastern Cape	33.654027°	25.246111°	<i>C. nanum</i>	1	1	0	1
14	Forest Swamp, Mkambati N.R	B	Eastern Cape	31.296530°	29.975944°	<i>C. nanum</i>	12	12	0	1
15	Fort Fordyce	A	Eastern Cape	32.678000°	26.511902°	<i>C. nanum</i>	2	2	1	1
16	Goso	B	Eastern Cape	31.433700°	29.633680°	<i>C. nanum</i>	5	5	1	1
17	Hluleka N.R	C	Eastern Cape	31.818080°	29.300240°	<i>C. nanum</i>	5	5	1	1
18	Hole in wall	C	Eastern Cape	32.037500°	29.108333°	<i>C. nanum</i>	1	1	0	1
19	Hogsback	A	Eastern Cape	32.606200°	26.961930°	<i>C. nanum</i>	2	2	1	1
20	Katberg	A	Eastern Cape	32.473600°	26.672510°	<i>C. nanum</i>	4	4	1	1
21	Kobolo	C	Eastern Cape	32.302780°	28.870350°	<i>C. nanum</i>	2	2	1	1
22	Kubusi	A	Eastern Cape	32.558684°	27.315337°	<i>C. nanum</i>	2	2	1	0
23	Lovemore Heights	B	Eastern Cape	34.002222°	25.527250°	<i>C. nanum</i>	1	1	0	1
24	Lubanzi	C	Eastern Cape	32.060510°	29.081159°	<i>C. nanum</i>	11	11	1	1
25	Main beach road, Mkambati N.R	C	Eastern Cape	31.307355°	29.976235°	<i>C. nanum</i>	3	3	1	1
26	Manubi	C	Eastern Cape	32.453375°	28.607377°	<i>C. nanum</i>	6	6	1	1
27	Mbotyi campsite	C	Eastern Cape	31.428000°	29.726150°	<i>C. nanum</i>	1	1	1	1
28	Mbotyi quarry	C	Eastern Cape	31.451467°	29.732108°	<i>C. nanum</i>	1	1	0	1

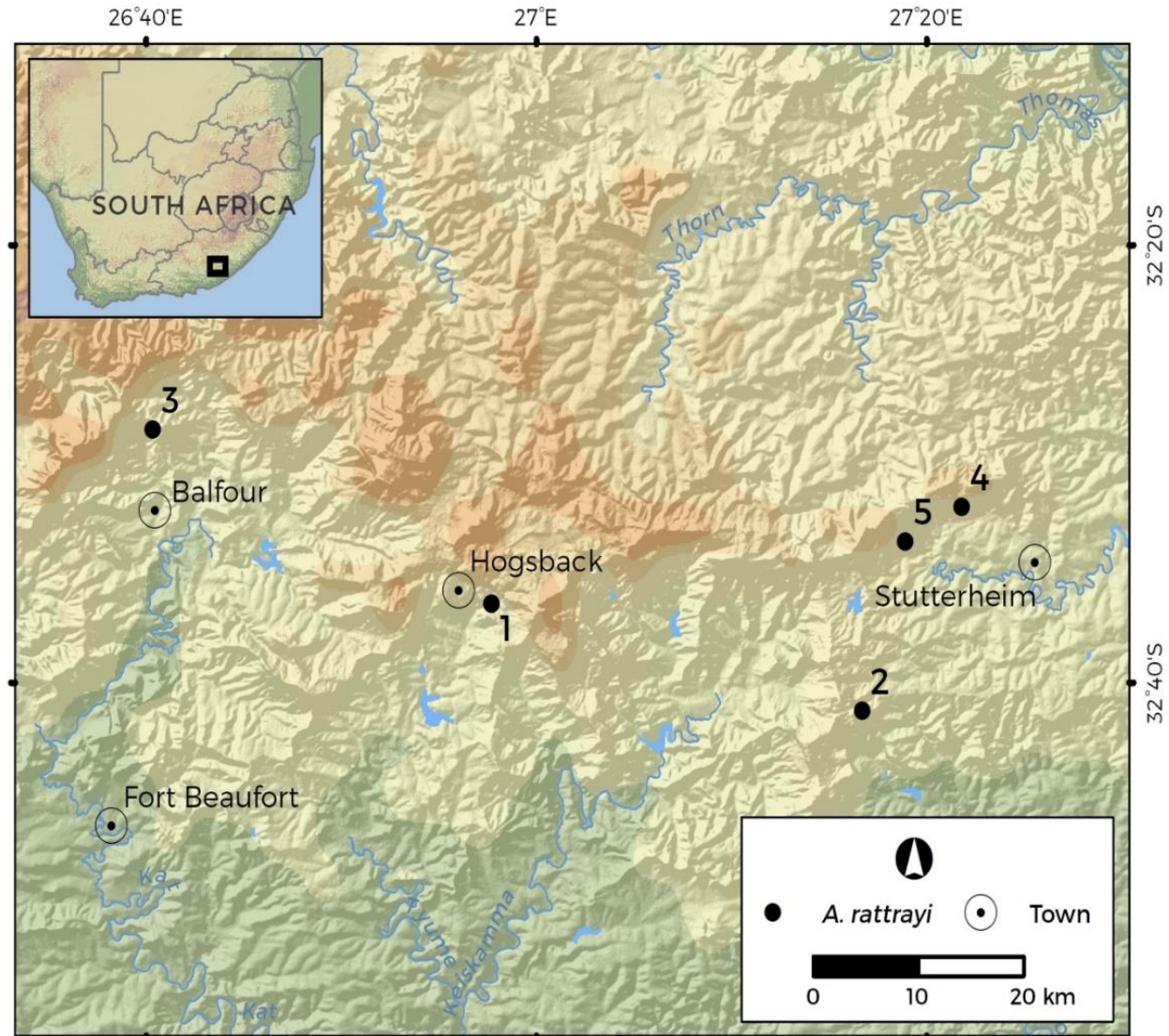
## Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.

**Table 1:** continues

N	Locality	Forest Type		GPS Coordinates		Species	Sequences generated per loci			
		A, B, C	Province	Latitude	Longitude		16S rRNA	Cyt <i>b</i>	RAG1	Rhodopsin
29	Mpofu N.R	B	Eastern Cape	32.598028°	26.571841°	<i>C. nanum</i>	2	2	0	0
30	Nature's Valley	B	Western Cape	33.976471°	23.561060°	<i>C. nanum</i>	2	2	0	1
31	NMU S.campus	B	Eastern Cape	34.008268°	25.666600°	<i>C. nanum</i>	1	1	0	1
32	Ntywenka	B	Eastern Cape	31.162206°	28.585390°	<i>C. nanum</i>	3	3	0	1
33	Nqadu	B	Eastern Cape	31.415034°	28.734500°	<i>C. nanum</i>	18	18	1	1
34	Otto du Plessis Pass	B	Eastern Cape	31.229450°	27.515440°	<i>C. nanum</i>	1	1	0	1
35	Silaka N.R	C	Eastern Cape	31.655310°	29.504570°	<i>C. nanum</i>	2	2	0	1
36	Superbowl, Mkambati	C	Eastern Cape	31.294009°	29.929676°	<i>C. nanum</i>	1	1	1	1
37	The Island N.R	B	Eastern Cape	33.979184°	25.372133°	<i>C. nanum</i>	5	5	0	0
38	Krantzkloof N.R	B	Kwa-Zulu Natal	29.772500°	30.830556°	<i>A. wahlbergii</i>	3	3	0	0
39	Nkandla N.R	B	Kwa-Zulu Natal	28.622500°	31.089444°	<i>A. wahlbergii</i>	4	4	0	0
40	Pinetown	B	Kwa-Zulu Natal	29.816667°	30.850000°	<i>A. wahlbergii</i>	1	1	0	0
<b>Total sequences generated</b>							206	206	25	33

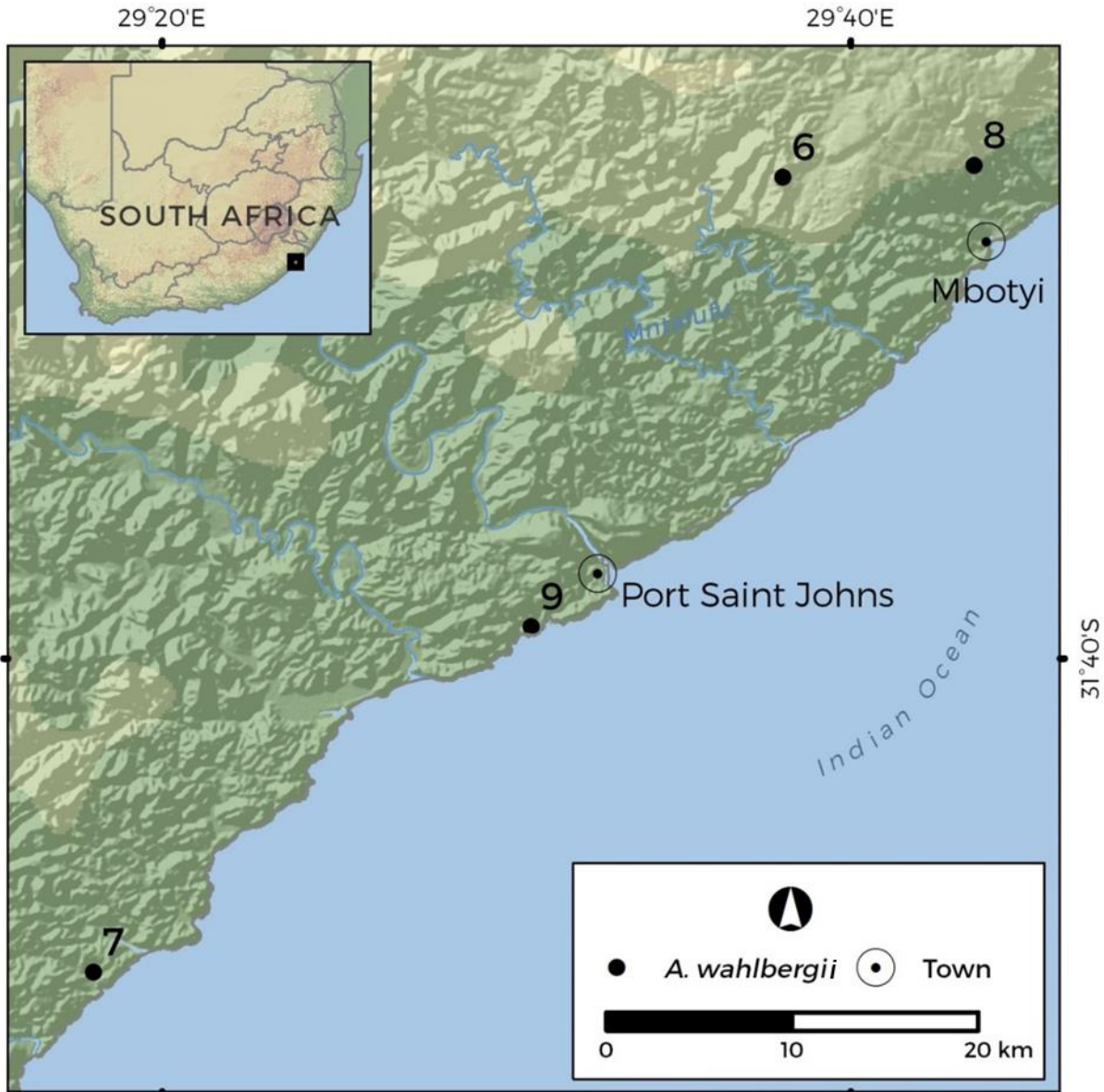
\*NMU S.campus= Nelson Mandela University South Campus

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**Figure 1:** Localities where *Anhydrophryne rattrayi* was collected in the Amatole Mountains of the Eastern Cape Province, South Africa. Solid black circles and numbers correspond to locations where *A. rattrayi* populations were sampled (Table 1). The open circles with a black dot represent the closest towns.

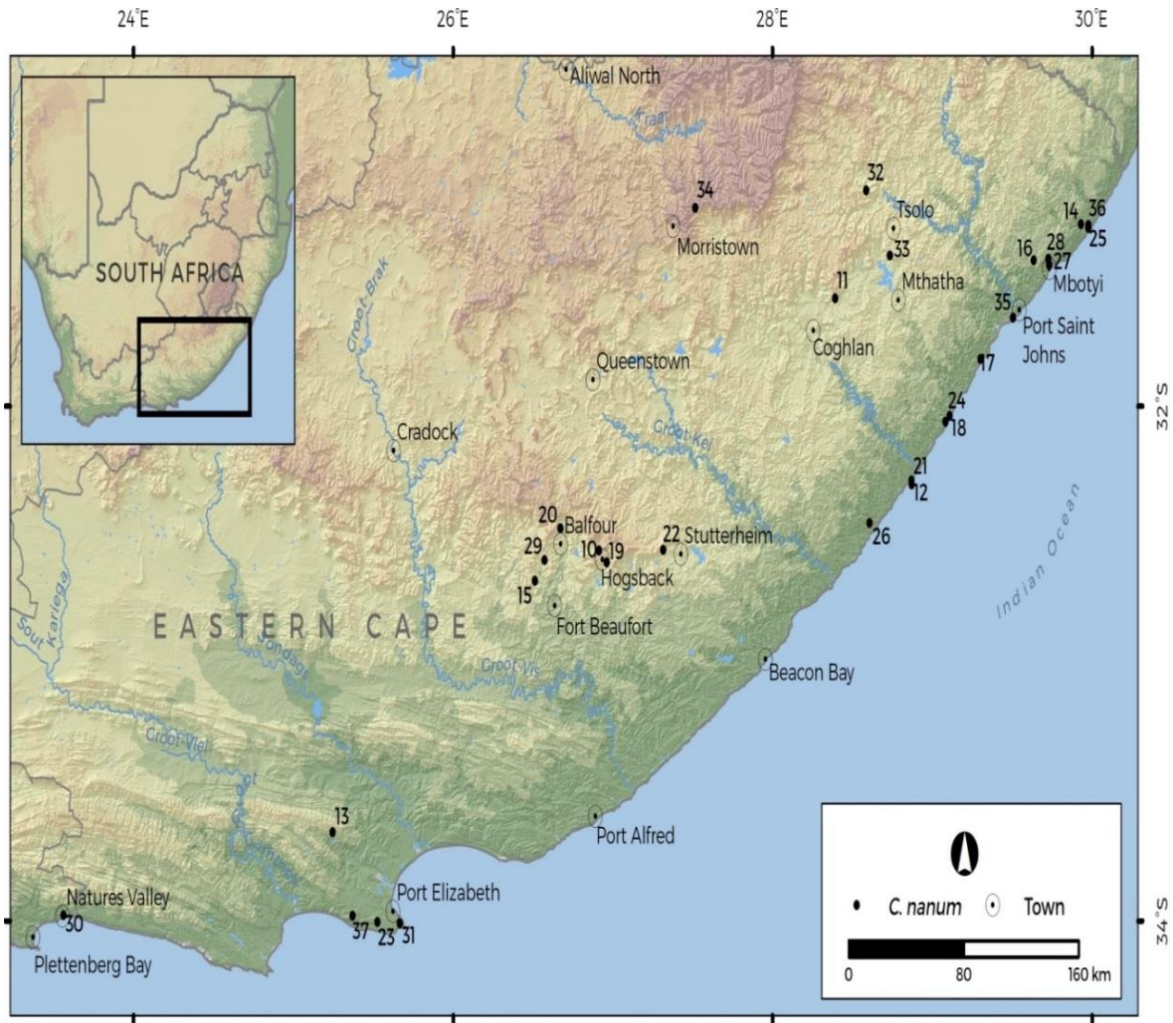
Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.



**Figure 2:** Localities where *Arthroleptis wahlbergii* was collected in the Eastern Cape Province, South Africa. Solid black circles and correspond to locations where *A. wahlbergii* populations were sampled (Table 1). The open circles with a black dot represent the closest towns. Localities from KwaZulu-Natal Province forests were not plotted.



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**Figure 3:** Localities where *Cacosternum nanum* was collected in the Eastern Cape and Western Cape Provinces, South Africa. Solid black circles and numbers correspond to localities where *C. nanum* populations were sampled (Table 1). The open circles with a black dot represent the closest towns.

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### 2.3 DNA extraction, polymerase chain reaction (PCR) and sequencing

Total genomic DNA was extracted from alcohol preserved tissue (toe clippings or liver) using a Machery-Nagel (GmbH & Co. KG, Germany) DNA extraction kit following the manufacturers' protocol (genomic DNA from tissue protocol). Extracted DNA was stored at 4°C until required for PCR. Two partial mitochondrial loci, 16S rRNA and Cytochrome *b* (Cyt *b*) were selected (Table 2) given their previous extensive use in phylogeographic studies of anurans (Gutiérrez-García and Vázquez-Domínguez, 2011; van der Meijden *et al.*, 2011; Zhang *et al.*, 2013; Barej *et al.*, 2015; Portillo *et al.*, 2015; Bittencourt-Silva *et al.*, 2016; Portik and Blackburn, 2016). However, mtDNA application is not without limitations (Avice, 2000; Hurst and Jiggins, 2005; Godinho *et al.*, 2008; Marshall *et al.*, 2011; Lapinski *et al.*, 2016). These limitations include being maternally inherited which poses a sex bias in fitness or dispersal behavior (Sato and Sato, 2013); it is highly variable and exhibits low intraspecific divergence (Avice, 2000; Dolman and Joseph, 2012). The inclusion of nuclear DNA (nuDNA) in the dataset has been known to counteract the limitations of haploid (mtDNA) molecule use (Edwards and Beerli, 2000; Zhang and Hewitt, 2003) in genetic studies. Thus, two nuclear gene loci, reactivation combination gene 1 (RAG1) and Rhodopsin (Rhod) were tested (Table 2). A single specimen per locality was sequenced for the two nuclear loci after preliminary analysis revealed low genetic variation for both loci within one locality. For each PCR, a 25 µl reaction was conducted that contained 14.9 µl millipore water, 3.5 µl of 2mM MgCl<sub>2</sub>, 2.5 µl of the 1 x reaction buffer solution, 0.5 µl of each primer pair, 0.5 µl of 0.1mM dNTPs, 0.1 µl of 0.5u Super-Therm BioTaq DNA polymerase (Super-Therm, JMR Holdings, London, United Kingdom) and 1.5 µl of template DNA.

To determine if PCR reagents were not contaminated, a negative control of all the reagents excluding the DNA template was also included in the PCR. Primers and detailed PCR conditions are provided in (Table 2). Ethidium bromide (0.5 µl/ml) stained agarose gels (1%) were used to verify amplification success. PCR products were dyed and loaded in agarose gels and ran in TBE buffer alongside a 1kB DNA ladder, electrophoresed at 90 V for 2-3 hours and viewed under ultra violet (UV) light. The gel bands of DNA were excised, and the DNA extracted and purified using a Bioflux purification kit (Bioer Technology Co, Ltd), following the manufacturers' protocol. The product was sent to the Central Analytical Facility (CAF) at Stellenbosch

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University for sequencing. Sequence electropherograms were visualized, edited and aligned manually in Sequence Navigator (Applied Biosystems, Foster City, CA, USA). Newly generated sequences will be deposited in GenBank and assigned Accession numbers before publishing this work. All sequences generated for all loci used were verified to be the correct species by running them in GenBank using BLAST (<http://blast.ncbi.nlm.nih.gov>), and to obtain some outgroups to root the trees. Additional sequences were kindly provided by Mr Werner Conradie, Curator at the Port Elizabeth Museum, Bayworld (see Appendix 1 for further information).

For data preparation, best fit models of sequence evolution were determined under the Akaike Information Criterion (AIC) in MrModel Test v2.3 (Nylander *et al.*, 2004) (Table 3) and preferred over the hierarchical implementation of the likelihood ratio test (hLRT) due to its computational strengths (Ripplinger and Sullivan, 2008). Genes were run separately to determine model selection then combined for the concatenated mtDNA dataset.

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**Table 2:** List of primers and PCR conditions with corresponding references used for molecular analysis in the present study. PCR conditions start with temperature (°C) of each step followed by the time in minutes and seconds and the number of cycles ran.

Gene	Primer name	Sequence 5' → 3'	PCR conditions	Sources
16S rRNA	16SA	CGCCTGTTTATCAAAAACAT	94°C (4mins), [94°C (30secs), 48°C (35secs), 72°C (45secs) x34 cycles], 72°C (10mins)	(Palumbi <i>et al.</i> , 1991)
	16SB	CCGGTCTGAACTCAGATCACGT	94°C (4mins), [94°C (30secs), 48°C (35secs), 72°C (45sec) x34 cycles], 72°C (10mins)	(Palumbi <i>et al.</i> , 1991)
Cyt <i>b</i>	Cyt B-CBJ10933	TATGTTCTACCATGAGGACAAATATC	94°C (4mins), [94°C (30secs), 48°C (35secs), 72°C (45secs) x34 cycles], 72°C (10mins)	(Bossuyt and Milinkovitch, 2000)
	Cyt <i>b</i> -C	CTACTGGTTGTCCTCCGATTCATGT	94°C (4mins), [94°C (30secs), 48°C (35secs), 72°C (45secs) x34 cycles], 72°C (10mins)	(Bossuyt and Milinkovitch, 2000)
RAG1	RAG1 MartF1	AGCTGCAGYCARTAYCAYAARATGTA	94°C (2mins), [94°C (1min), 50°C (1min), 72°C (1min) x36 cycles], 72°C (7mins)	(Chiari <i>et al.</i> , 2004)
	RAG1 AmpR1	AACTCAGCTGCATTKCCAATRTCA	94°C (2mins), [94°C (1min), 50°C (1min), 72°C (1min) x36 cycles], 72°C (7mins)	(Pramuk <i>et al.</i> , 2008)
Rhodopsin	Rhod-ma	AACGGAACAGAAGGYCC	94°C (2mins), [94°C (30secs), 51°C (35secs), 72°C (45secs) x36 cycles], 72°C (10mins)	(Bossuyt and Milinkovitch, 2000)
	Rhod-md	GTAGCGAAGAARCCTTC	94°C (2mins), [94°C (30secs), 51°C (35secs), 72°C (45) x36cycles], 72°C (10mins)	(Bossuyt and Milinkovitch, 2000)

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**Table 3:** Substitution models selected in MrModeltest for the 16S rRNA and Cyt *b* loci for *Anhydrophryne rattrayi*, *Arthroleptis wahlbergii* and *Cacosternum nanum*. An asterisk \* indicates a ti/tv ratio of 10.77.

Gene	bp	Species	Model selected			Invariable sites I	Variable sites G	Model parameters			
			Substitution model	Log likelihood - InL	AIC			Rate matrix	Base frequency		%
16S rRNA	505	<i>A. rattrayi</i>	GTR+G	1089.47	2196.94	-	0.12	A-C	1.16	A	30.72
								A-G	3.51	C	22.22
								A-T	2.20	G	20.42
								C-G	0.00	T	26.63
								C-T	7.65		
								G-T	1.00		
	520	<i>A. wahlbergii</i>	TrN+I	1295.45	2600.90	0.71	-	A-C	1.00	A	34.12
								A-G	3.78	C	24.23
								A-T	1.00	G	17.18
								C-G	1.00	T	24.47
								C-T	8.93		
								G-T	1.00		
523	<i>C. nanum</i>	TrN+I	1043.20	2096.40	0.75	-	A-C	1.00	A	30.03	
							A-G	2.42	C	25.11	
							A-T	1.00	G	20.09	
							C-G	1.00	T	24.78	
							C-T	7.26			
							G-T	1.00			
Cyt <i>b</i>	505	<i>A. rattrayi</i>	GTR+I+G	1829.52	3679.05	0.45	0.65	A-C	0.00	A	24.37
								A-G	8.03	C	11.77
								A-T	1.10	G	37.22
								C-G	0.07	T	26.64
								C-T	3.41		
								G-T	1.00		
	543	<i>A. wahlbergii</i>	TrN+I	1600.39	3210.77	0.61	-	A-C	1.00	A	30.35
								A-G	6.81	C	9.95
								A-T	1.00	G	33.05
								C-G	1.00	T	26.65
								C-T	27.35		
								G-T	1.00		
531	<i>C. nanum</i>	HKY 85+G	1255.77	2521.56	-	0.49	*		A	34.02	
									C	11.38	
									G	27.97	
									T	26.63	

## 2.4 Phylogenetic analysis

For the present study, phylogenetic analysis was performed on a combined dataset of the targeted mitochondrial (16S rRNA, Cyt *b*) loci as there are several drawbacks to using data generated from a single gene in making substantial inferences at population level (Crandall and Templeton, 1993) and separately for the nuclear gene loci RAG1 and Rhod. For preliminary phylogenetic analysis, Maximum Parsimony (MP) analyses was ran in PAUP\* v4.0 (Swofford, 2002) to have a general estimate of genealogical relationships among the two mtDNA. Two robust methods of phylogenetic reconstruction, Maximum Likelihood (ML) analysis and Bayesian Inferences (BI) were subsequently conducted. Evolutionary history using the ML method was inferred based on the General Time Reversible model (Nei and Kumar, 2000), executed in MEGA v7.0 (Kumar *et al.*, 2016). In the ML analysis (Swofford, 2002) initial tree(s) under a heuristic search strategy were obtained automatically through the application of Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and subsequently selecting the topology with superior log likelihood value. A discrete Gamma distribution for *A. rattrayi*, *A. wahlbergii* and *C. nanum* was used to model evolutionary rate differences among sites [5 categories (+G, parameter 3.99, 0.47 and 0.15)], respectively.

Bayesian inference is considered a robust phylogenetics analytical tool as it has computational advantages such as the inclusion of priors and a tool to specify the evolutionary model (Huelsenbeck and Ronquist, 2001). Thus, BI as implemented in MRBAYES v3.2 (Ronquist *et al.*, 2012) was conducted based on posterior probabilities of phylogenetic trees (Huelsenbeck and Ronquist, 2001). Analysis was run for 20 000 000 generations, with one tree saved every 2000 generations. Convergence of the Bayesian runs was checked in Tracer v1.5 (Rambaut and Drummond, 2009). The first 10 000 trees generated were determined as burn-in and thus discarded. Only trees sampled after this burn-in phase were used to ascertain posterior probabilities (bpp, branch-lengths and clades) by generating a consensus tree in MrBayes. The BI trees were viewed in FigTree v1.4.2 (Rambaut, 2009).

## 2.5 Phylogeographic reconstruction

Population genetic structure was explored using the rapidly evolving Cyt *b* locus in Arlequin v3.0 (Excoffier *et al.*, 2005) to calculate summary statistics of all derived lineages within the

three taxa such as number of haplotypes ( $N_h$ ), number of polymorphic sites ( $N_p$ ), gene diversity, and nucleotide diversity ( $\Pi$ ). To investigate the ratio of genetic variation within the three-study species, pairwise genetic distances ( $F_{ST}$ ) among populations were calculated in Arlequin v3.0 using 1000 permutations (Excoffier *et al.*, 2005) at 95% significance level. Pairwise genetic distances ( $F_{ST}$ ) was chosen for determining genetic differentiation among populations because it effectively summarizes the effects of population structure (Whitlock, 2011). To determine the extent of genetic variation among and within populations or hierarchical population structure, an analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) was performed by forming geographic groups from pooling populations from different sampling localities.

Additionally, uncorrected pairwise sequence divergence values were calculated for the Cyt *b* locus using MEGA v7.0 (Kumar *et al.*, 2016). For *A. rattrayi*, the analysis involved 48 nucleotide sequences (including the two outgroup sequences) with a total of 505 positions in the final dataset. For *A. wahlbergii*, the analysis involved 48 nucleotide sequences; with the inclusion of two outgroup taxa and eight KZN sequences from three locations to illustrate the sequence divergence difference between the Eastern Cape and KwaZulu-Natal Province specimens and the final dataset consisted of a total of 373 positions. The *Cacosternum nanum* dataset involved 116 nucleotide sequences including two from Western Cape Province whereas the other two represented the outgroup taxa, 531 positions were in the final dataset. All codon positions were included. All positions with less than 95% site coverage were eliminated. Thus, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

To examine the geographic distribution of genetic variation through the construction of haplotype phylogenies, TCS v1.2.1 (Clement and Posada, 2000; Clement *et al.*, 2000) was used. Under a statistical parsimony framework (Templeton *et al.*, 1992) at 95% confidence interval, genealogical relationships between the varying haplotypes were inferred. Given the effectiveness of haplotype networks in depicting relationships at intraspecific level (Vilá *et al.*, 1999), their incorporation to support lineage relationships inferred from phylogenetic trees is essential.

## 2.6 Demographic history

To determine the history of an effective population i.e. unmask population demography and to test whether populations are in equilibrium, Fu's  $F_s$  (Fu, 1997) neutrality test was used. Fu's  $F_s$  ensures that the observed sequence polymorphism in a lineage follows that of a neutral model. Fu's  $F_s$  were generated in Arlequin v3.1 using parametric bootstrapping values of 10,000 replicates (Felsenstein, 1981). Negative and statistically significant Fu's  $F_s$  indicates an excess of alleles generated from a recent population expansion, genetic hitch-hiking or under purifying selection (Fu, 1997). This is characterized by low frequency haplotypes and low divergence, low nucleotide diversity, high haplotype diversity and high polymorphic sites. A positive Fu's  $F_s$  indicates the presence of low frequency alleles expected from a recent population bottleneck (population recovering), statistically significant at  $p < 0.02$ . Although less sensitive to recent population expansion than Fu's  $F_s$ , Tajima's  $D$  (Ramos-Onsins and Rozas, 2002) was also included to corroborate Fu's  $F_s$  indices. The same principles apply for Tajima's  $D$ ; negative and statistically significant ( $p < 0.10$ ) values indicate a populations' demographic expansion history (Tajima, 1989a).

To further explore demographic histories of the three anuran species, a mismatch distribution analysis (Ramos-Onsins and Rozas, 2002) was run in Arlequin v3.1. Sum of squared deviations (SSD) and raggedness ( $r$ ) measures are used to as indices in population demography (Rogers and Harpending, 1992). A low and non-significant SSD and  $r$  value supports demographic expansion of a population whereas statistically significant and high values support population reduction or subdivision, migration or a recent bottleneck (Rogers and Harpending, 1992).

## 2.7 Divergence Time Estimates

No fossil records or rates of diversification are known for the three species. Further, previous studies have used secondary calibration points such as splits within Ranoidea (e.g. van der Meijden *et al.*, 2005) and calibration points constrained to the MRCA at family level (Roelants *et al.*, 2007; Loader *et al.*, 2014; Portik and Blackburn, 2016). However, the application of such secondary calibrations in estimating divergences and use of higher taxonomic scales for population level analyses has been debated in the past (Hug and Roger, 2007; Forest, 2009;



Sauquet, 2013; Hipsley and Müller, 2014). Thus, the present study employed molecular clocks for the mitochondrial loci 16S rRNA and *Cyt b* to estimate the divergence dates for the lineages of *Anhydrophryne rattrayi*, *Arthroleptis wahlbergii* and *Cacosternum nanum* in the program BEAST v1.8.1 (Drummond *et al.*, 2012). For the molecular clock rates, the present study applied a strict molecular clock model using a coalescent tree prior to the mitochondrial gene datasets. Analyses was run for 10 000 000 generations for the two forest specialists, *A. rattrayi* and *A. wahlbergii* whereas 6 000 000 generations were run for *C. nanum*, sampling every 1000 generations given the magnitude of data as well as to obtain sufficient effective sampling size (ESS). The present study assumed a range of substitution rates from 0.60% to 1.00% per million years for *Cyt b* and 0.20% to 0.30% per million years for the 16S rRNA locus, based on rates published for anurans (Macey *et al.*, 2001; Fouquet *et al.*, 2009; Pröhl *et al.*, 2010; Portillo *et al.*, 2015; Larson *et al.*, 2016). Initially, the substitution rates for each locus were run independently. However, due to different mutation rates of the two loci, discordance between the divergences was observed in the species' phylogenies. Thus, for final analysis, the substitution rates for the two loci were combined and an average was calculated. The program BEAUti (Drummond *et al.*, 2012) in BEAST was used to determine the Markov Chain Monte Carlo (MCMC) analysis. Tracer v1.5 (Rambaut and Drummond, 2009) was used to assess ESS of parameters investigated. Sufficient ESS values >200 were obtained for all parameters. The first 10% trees generated were considered as burn-in and thus discarded using LogCombiner v1.7.5 in the BEAST package. Using the posterior mean node heights for clades, the most credible tree was summarized using Tree Annotator v1.7.5 (Drummond *et al.*, 2012) and viewed in FigTree v1.4.2.

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# **CHAPTER THREE**

## **RESULTS**

## Chapter 3

### Results

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### 3. Results

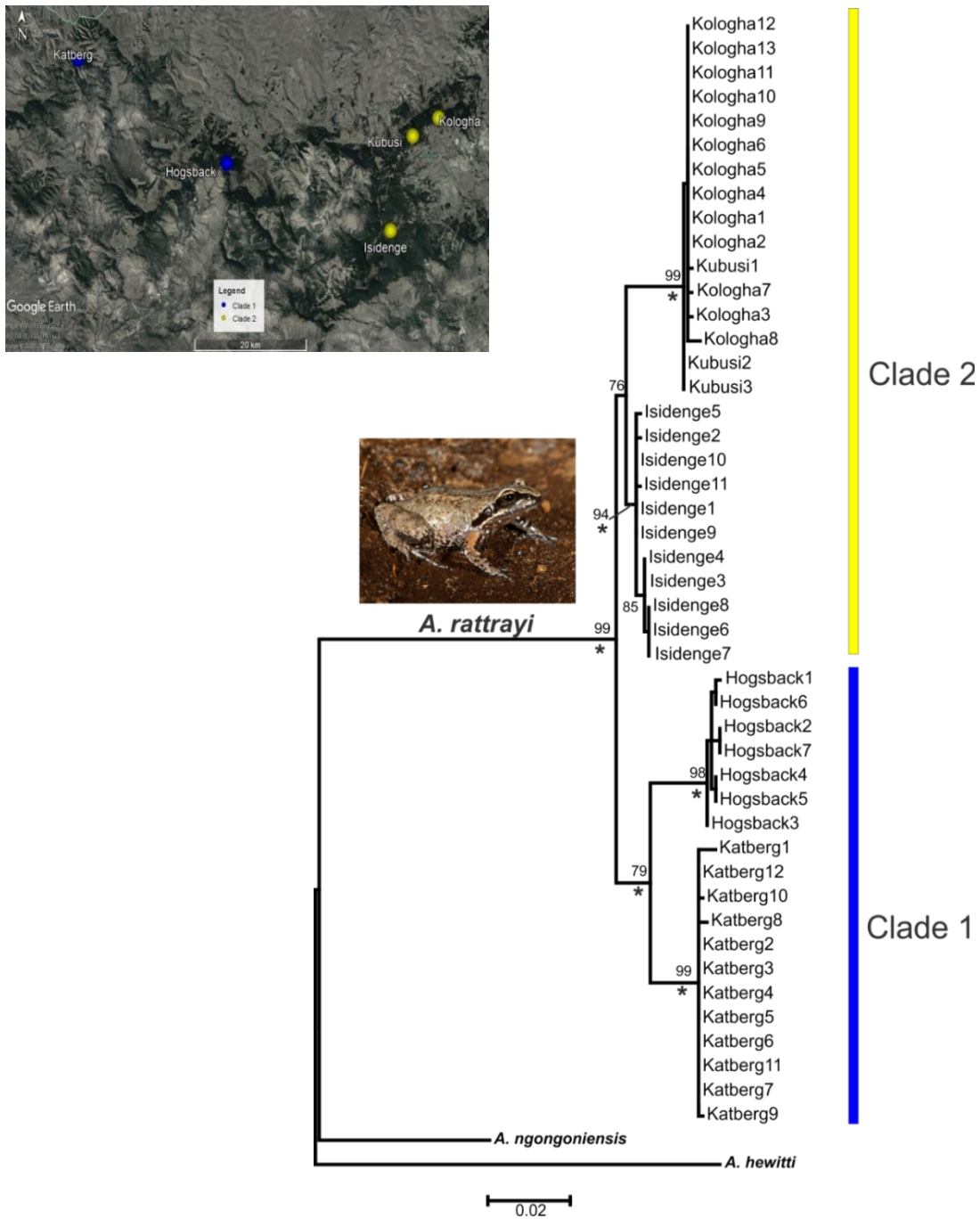
#### 3.1 Combined mtDNA tree topologies

Since the two loci (16S rRNA and Cyt *b*) are maternally inherited and linked on the mitochondria, the two loci were combined, and analysis was conducted on a combined mtDNA dataset. Evolutionary relationships were inferred by using a total of 1010bp, 1086bp and 1054bp of the combined mtDNA data for *A. rattrayi*, *A. wahlbergii* and *C. nanum*, respectively. As the BI and ML analyses retrieved similar topologies for all three species, only the ML tree topologies are presented.

##### 3.1.1 *Anhydrophryne rattrayi*

The combined mtDNA topology retrieved a monophyletic *A. rattrayi* (Fig. 4). Two genetically distinct and statistically well supported clades observed, exhibited strong geographical structure. Clade 1 comprised specimens from Katberg, north-west of the Amatole Mountains, sister to specimens from Hogsback to the south-west of the Amatole Mountains. Clade 2 comprised specimens from the east of the Amatole Mountains and included specimens from Isidenge to the south-east sister to specimens from Kologha and Kubusi. The uncorrected intraspecific pairwise sequence divergence values between *A. rattrayi* sample localities for the Cyt *b* locus ranged from 0.20% to 6.70% with a mean of 3.60% whereas within populations, the uncorrected pairwise sequence divergence was <1.00%. The uncorrected sequence divergence values between clade 1 and clade 2 for the Cyt *b* locus was 5.30% whereas within clade 1, the sequence divergence was 1.70% and within clade 2, the sequence divergence was 2.40%.

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**Figure 4:** ML tree topology of *Anhydrophyrne rattrayi* based on two mtDNA (16S rRNA and Cyt *b*) loci. Numbers above and below nodes indicate nodal support for bootstrapping and posterior probabilities (pP), respectively. Nodes with >0.95 pP are marked with an asterisk. Coloured lines correspond to geographically distinct interrelated clades. Insert: Map indicating geographical localities of clades 1 and 2, coloured dots corresponding to clades on tree topology.

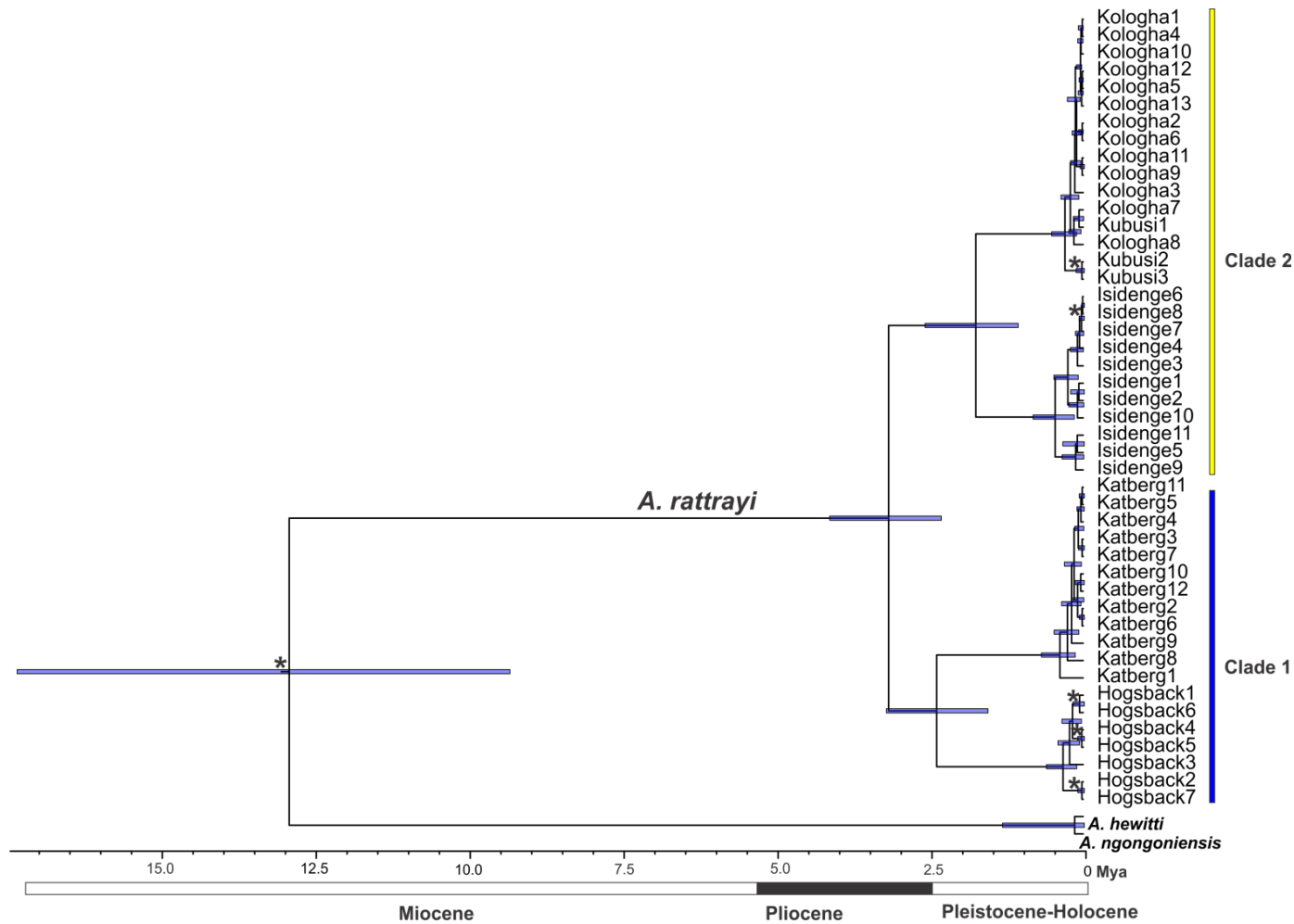
The divergence time analyses suggest that *A. rattrayi* diverged from its sister taxa during the Late Pliocene [mean: 3.23 mya, 95% highest posterior density (HPD): 2.32-4.14 mya] (Fig. 5). The two *A. rattrayi* clades diversified at different time scales. Clade 1 diversified during the Late Pliocene [mean: 3.18 mya, 95% highest posterior density (HPD): 1.57-3.22 mya] while clade 2 diversified during the Early Pleistocene [mean: 2.38 mya, 95% highest posterior density (HPD): 1.08-2.59 mya] (Fig. 5).

### 3.1.2 *Arthroleptis wahlbergii*

The combined mtDNA topology retrieved a monophyletic *A. wahlbergii* (Fig. 6). Three genetically distinct and statistically well supported clades were observed. Clade 1 comprised specimens from Krantzkloof N.R and Pinetown, sister to specimens from Nkandla N.R exclusively from the KwaZulu-Natal Province. Clade 2 comprised specimens from Mbotyi, sister to clade 3 which comprised specimens from Hluleka N.R, sister to specimens from Silaka N.R and Goso, Eastern Cape Province. The uncorrected intraspecific pairwise sequence divergence values between the *A. wahlbergii* Eastern Cape Province sampled populations for the Cyt *b* locus ranged from 0.30% to 6.70% with a mean of 3.30% whereas within populations, the uncorrected pairwise sequence divergence was <1.00%. The uncorrected sequence divergence values between clade 1 and clade 2 for the Cyt *b* locus was 12.20%, 11.70% between clade 1 and clade 3 whereas between clade 2 and clade 3, the sequence divergence was 5.40%. The uncorrected pairwise sequence divergence within clade 1 was 2.50%, and <1.00% for clades 2 and 3.

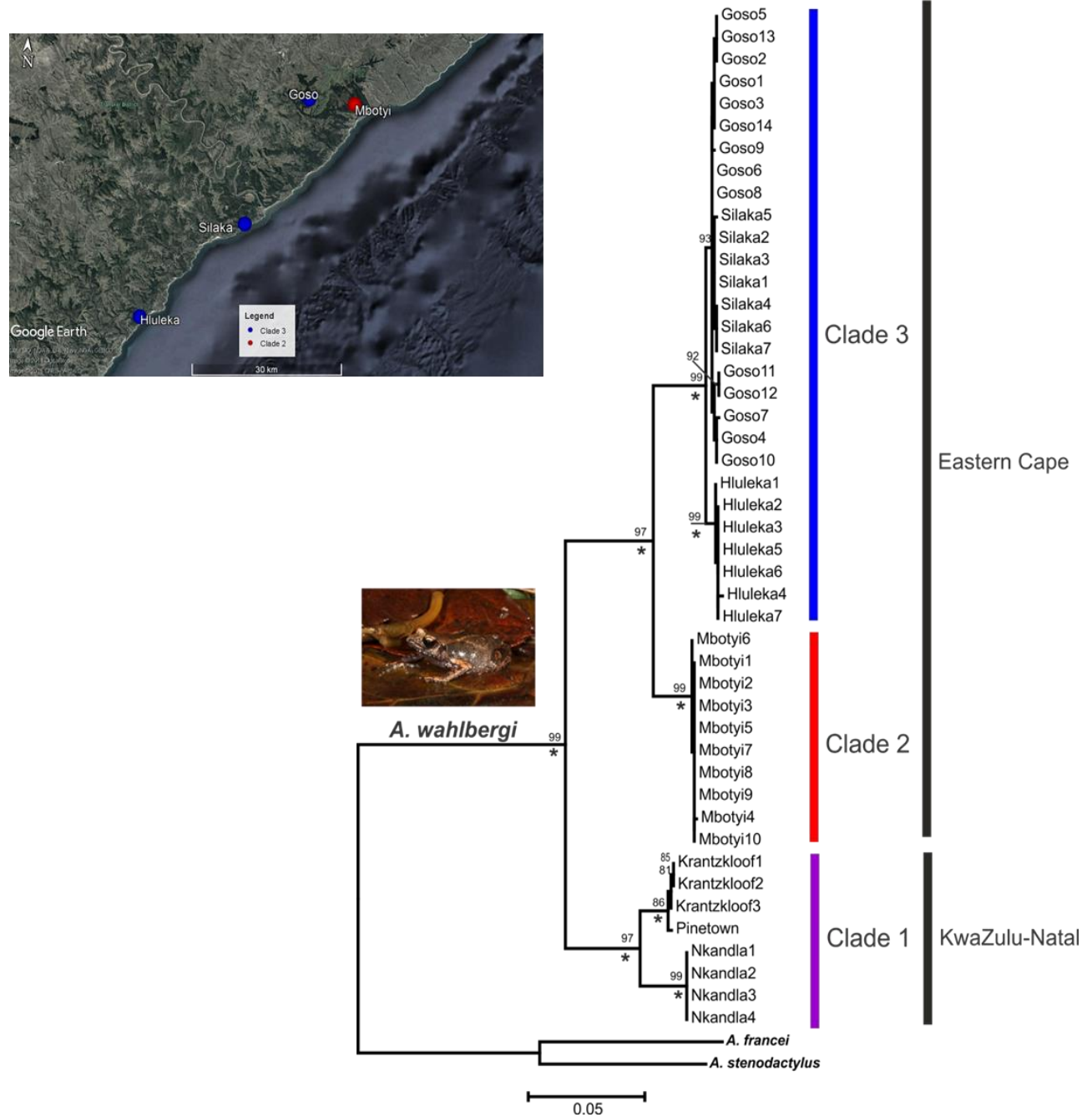
The divergence time analyses suggest that *A. wahlbergii* diverged from its sister taxa during the Middle Miocene [mean: 14.34 mya, 95% highest posterior density (HPD): 8.02-12.63 mya] (Fig. 7). The Eastern Cape clades diverged during the Late Miocene [mean: 5.76 mya, 95% highest posterior density (HPD): 3.01-5.50 mya]. Clade 1 (KwaZulu-Natal clade) diversified during the Early Pliocene [mean: 3.92 mya, 95% highest posterior density (HPD): 1.92-4.00 mya], clade 2 diversified during the Late Pleistocene [mean: 0.12 mya, 95% highest posterior density (HPD): 0.02-0.19 mya] while clade 3 diversified during the Middle Pleistocene [mean: 1.39 mya, 95% highest posterior density (HPD): 0.63-1.52 mya] (Fig. 7).

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**Figure 5:** Chronogram resulting from BEAST based on the combined substitution rates of the mtDNA (Cyt *b* and 16S rRNA) for *Anhydrophryne rattrayi*. Nodes with high bootstrap support >0.95 are indicated by an asterisk \*. Posterior estimates are provided along with bars on nodes representing the 95% highest posterior densities (HPD). Coloured clade lines correspond to distinct and geographically cohesive clades (Fig. 4).

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**Figure 6:** ML tree topology of *Arthroleptis wahlbergii* based on two mtDNA (16S rRNA and Cyt *b*) loci. Numbers above and below nodes indicate nodal support for bootstrapping and posterior probabilities, respectively. Nodes >0.95 pP are marked with an asterisk. Coloured lines correspond to geographically distinct interrelated clades. Insert: Map indicating geographical localities of clades 2 and 3, coloured dots corresponding to clades on tree topology.

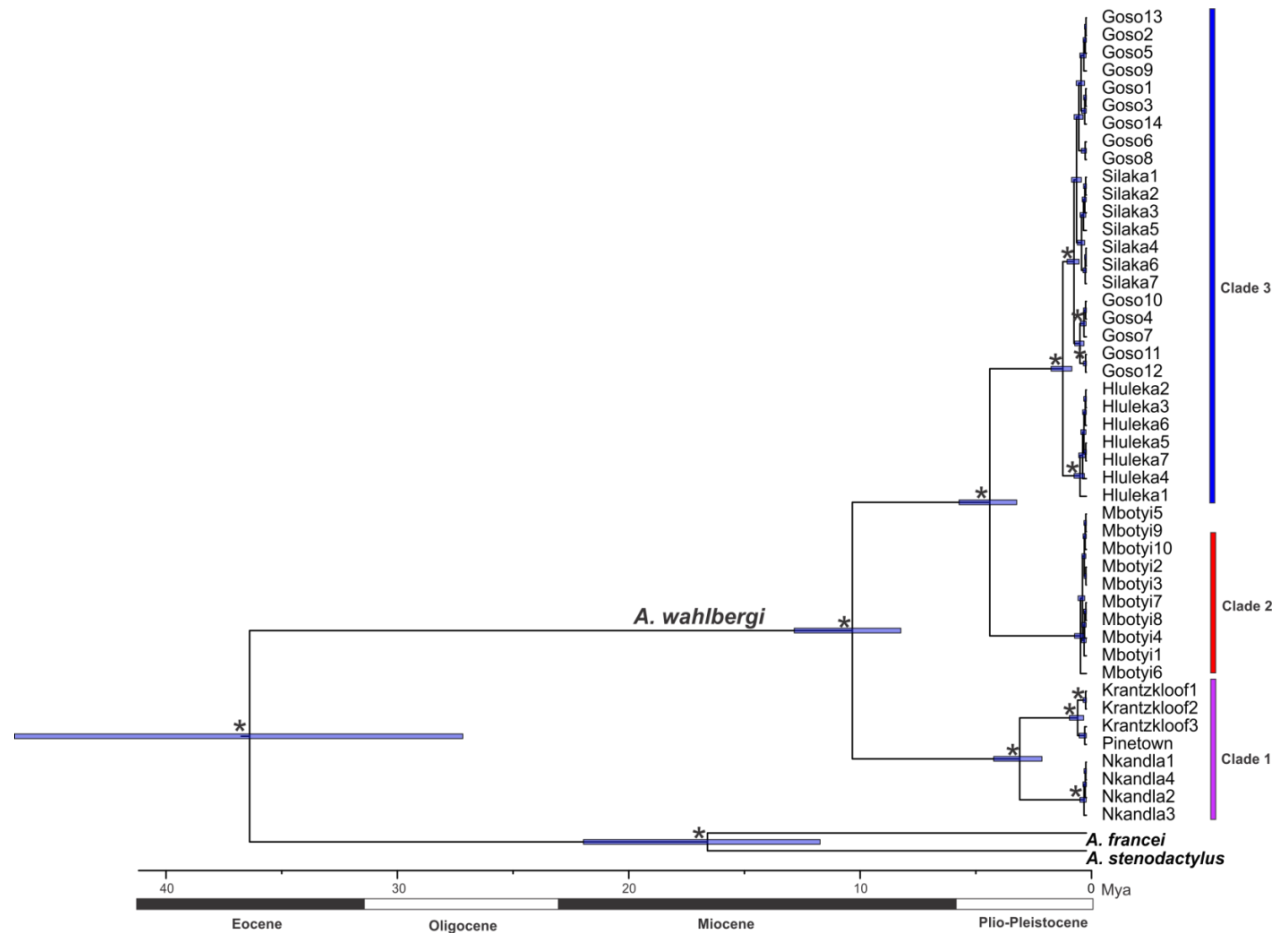
### 3.1.3 *Cacosternum nanum* (combined mtDNA topology not shown)

The combined mtDNA topology retrieved a monophyletic *C. nanum*. Specimens from the southernmost distribution of *C. nanum* collected within the study formed a well-supported clade (clade 1) comprising specimens from The Island N.R, Eels Cave, Lovemore Heights, NMU S.campus and Natures Valley, sister to a group of specimens from the remaining sample localities from the northern distribution range collected within the Eastern Cape Province. The uncorrected intraspecific pairwise sequence divergence between the *C. nanum* sample localities for the Cyt *b* locus ranged from <0.20% to 4.30% with a mean of 1.60% whereas within sampled sites, the range was low (<1.00%). Between clade 1 and the rest of the *C. nanum* sample localities (clade 2), the uncorrected sequence divergence was 3.10% whereas within clade 1, the sequence divergence was 0.70% and within the rest of the localities, the sequence divergence was 1.20%.

The divergence time analyses suggest that *C. nanum* diverged from its sister taxa during the Late Pliocene to Early Pleistocene [mean: 3.00 mya, 95% highest posterior density (HPD): 1.49-3.01 mya] (Fig. 8). The two *C. nanum* clades diversified at different time scales. Clade 1 diversified during the Middle Pleistocene [mean: 0.80 mya, 95% highest posterior density (HPD): 0.28-1.03 mya] while group 2 diversified during the Early Pleistocene [mean: 1.76 mya, 95% highest posterior density (HPD): 0.87-1.78 mya] (Fig. 8).

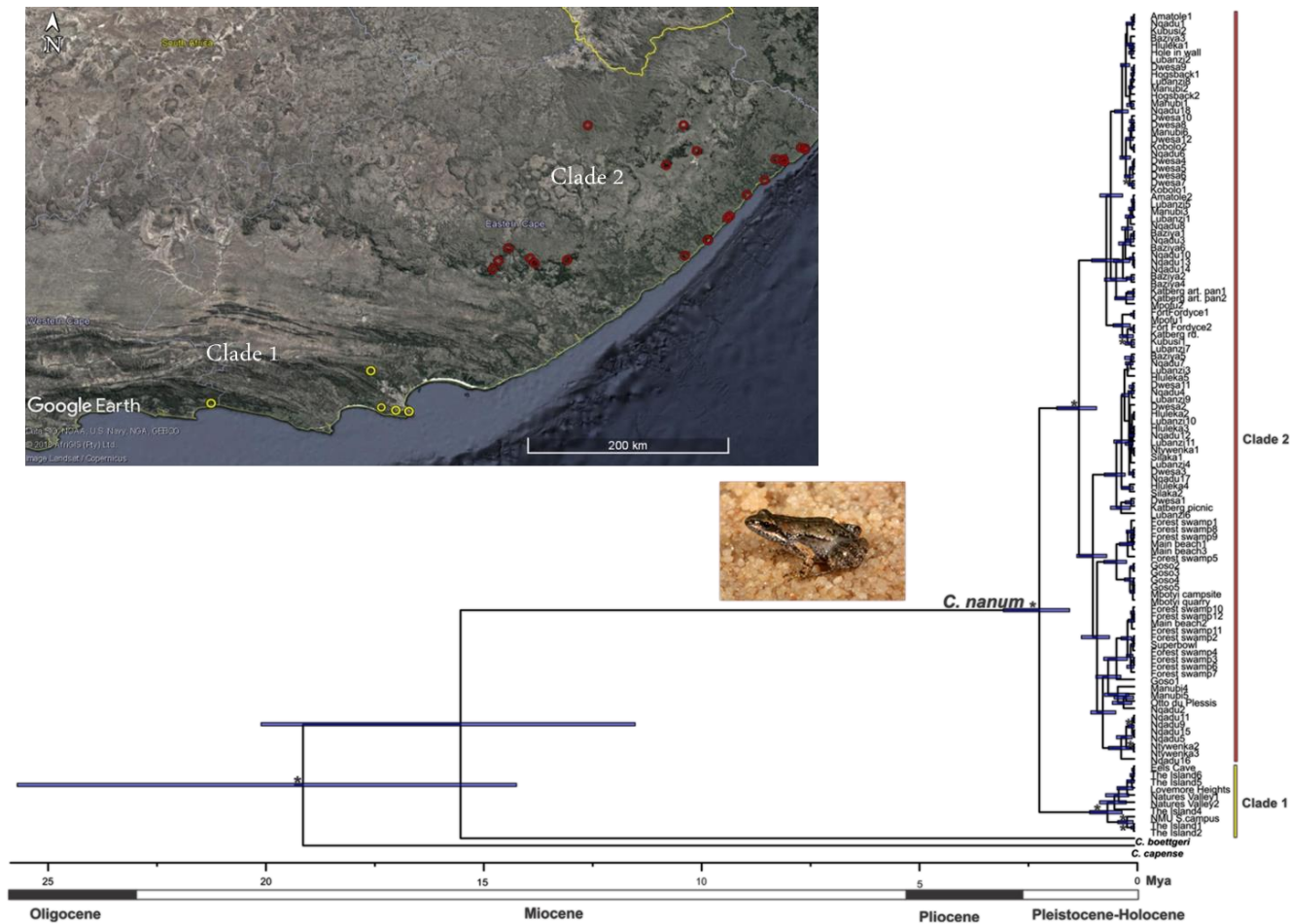


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**Figure 7:** Chronogram resulting from BEAST based on the combined substitution rates of the mtDNA (Cyt *b* and 16S rRNA) for *Arthroleptis wahlbergii*. Nodes with high bootstrap support  $>0.95$  are indicated by an asterisk \*. Posterior estimates are provided along with bars on nodes representing the 95% highest posterior densities (HPD). Coloured clade lines correspond to distinct, geographically cohesive clades (Fig. 6).

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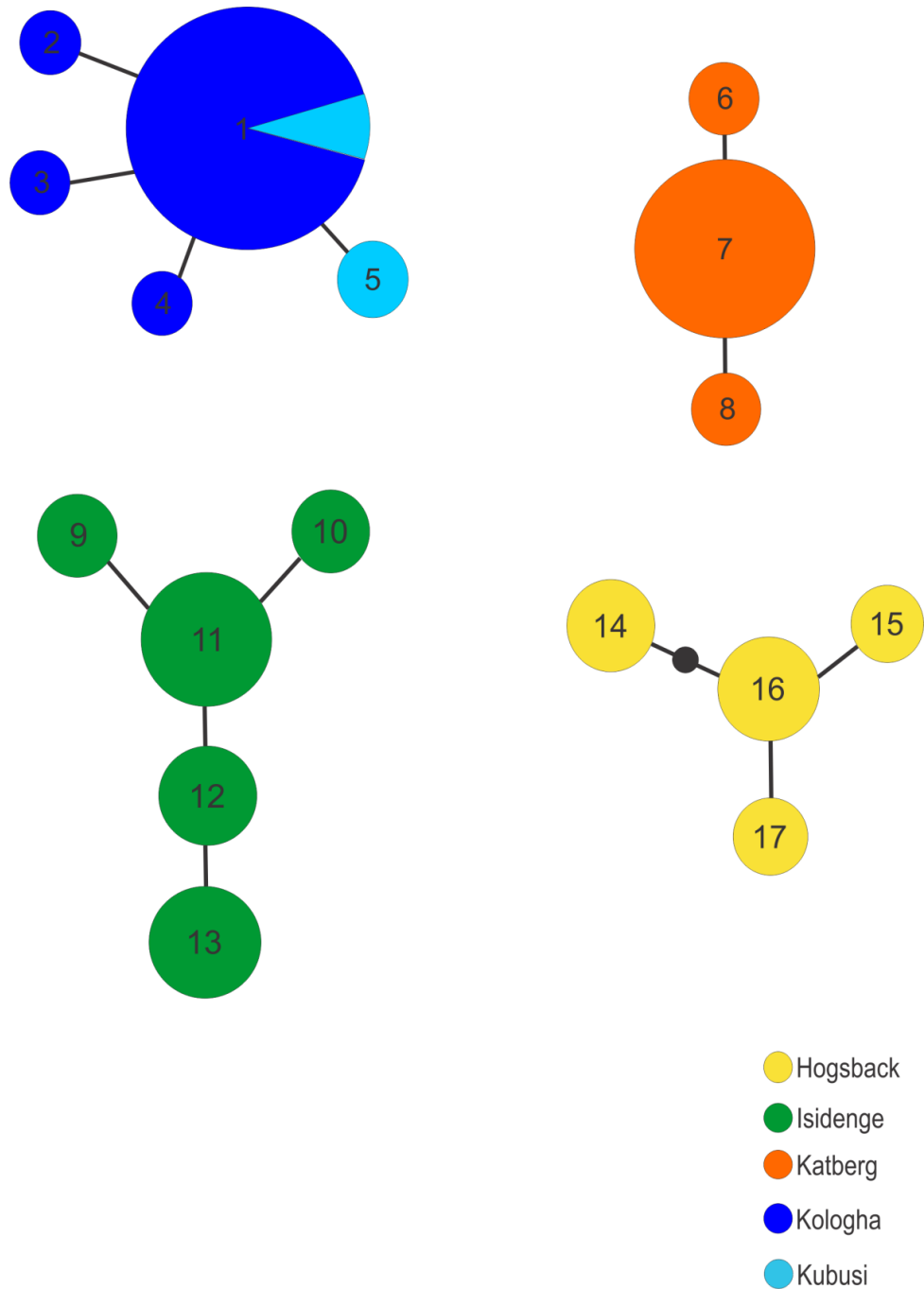
**Figure 8:** Chronogram resulting from BEAST based on the combined substitution rates of the mtDNA (Cyt *b* and 16S rRNA) for *Cacosternum nanum*. Nodes with high bootstrap support >0.95 are indicated by an asterisk \*. Posterior estimates are provided along with bars on nodes representing the 95% highest posterior densities (HPD). Coloured clade lines correspond to distinct, geographically cohesive clades. Insert: Map indicating geographical localities of clades 1 and 2, coloured dots corresponding to clades indicated on chronogram.

### 3.3 Phylogeographic analyses

#### 3.3.1 *Anhydrophryne rattrayi*

The TCS analyses retrieved a total of 17 Cyt *b* haplotypes for the 46 *A. rattrayi* specimens sequenced (Fig. 9). At 95% confidence intervals, no haplotypes were shared between sample localities except specimens from Kologha and Kubusi where haplotype 1 was shared (Fig. 9, Table 4). The number of polymorphic sites within sample localities was generally low (Table 5). Gene diversity was high suggesting strong genetic variation between the populations of *A. rattrayi*. Nucleotide diversity was low across all sampled localities (Table 5) and can be attributed to the small and fragmented populations of *A. rattrayi* restricted to high altitude habitats. Among *A. rattrayi* populations, 95.92% of the variation was present between sample localities (d.f. =4, sum of squares =392.93,  $V_a = 11.08$ ), whereas 4.08% of the variation was observed within sampled localities (d.f. =41, sum of squares =19.331,  $V_b = 0.47$ ). Statistically significant pairwise  $F_{ST}$  values (Table 6) indicate marked population genetic differentiation, except Kologha and Kubusi. All localities had statistically non-significant  $p$  values for both neutrality tests except Katberg and Kologha which had Tajima's  $D$  ( $p = 0.02$  and  $0.01$ ), respectively (Table 5). The mismatch distributions for the Cyt *b* locus for *A. rattrayi* indicated low and non-significant raggedness values and SSD ( $p > 0.05$ ) (Table 5) suggesting that the populations might not have experienced a sudden population expansion. However, a significant SSD value ( $p = 0.00$ ) found in Kologha suggests that this population might not have undergone a sudden population expansion.

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**Figure 9:** TCS haplotype networks of *Anhydrophryne rattrayi* (46 sequenced specimens) from five localities, based on 505bp of the *Cyt b* locus. Each circle represents one haplotype; number inside each haplotype represents haplotype number; size of circle is proportional to haplotype frequency and colour represents population/ sample locality corresponding to Fig. 1. The partitions within the circles represent the proportion of each sampled population within a haplotype. Black dots represent putative haplotypes that were unsampled or are missing.

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**Table 4:** Summary of the Cyt *b* haplotype frequencies between and within *Anhydrophryne rattrayi* sample localities (corresponds to Fig. 9).

Locality	Haplotypes																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 Isidenge									1	4	1	2	3				
2 Katberg						1	10	1									
3 Hogsback														2	3	1	1
4 Kologha	10	1	1	1													
5 Kubusi	1				2												

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**Table 5:** Summary list of the population parameters for each of the *Anhydrophryne rattrayi* sampled localities. N is the number of samples per locality, Nh is the number of haplotypes and Np is the number of polymorphic sites. The \* indicates  $p < 0.02$ ,  $< 0.10$ ,  $< 0.05$  for Fu's  $F_s$ , Tajima's  $D$  and mismatch distribution parameters SSD and  $r$ , respectively.

Locality	N	Nh	Np	Nucleotide diversity ( $\pm$ SD)		Gene/haplotype diversity ( $\pm$ SD)		Tajima's $D$	Fu's $F_s$	Mismatch distribution	
				SSD	$r$	SSD	$r$				
1 Isidenge	11	5	4	0.00	$\pm$ 0.00	0.82	$\pm$ 0.08	-0.05	-1.33	0.53	0.46
2 Katberg	12	3	3	0.00	$\pm$ 0.00	0.32	$\pm$ 0.16	-1.63*	-0.61	0.44	0.55
3 Hogsback	7	4	4	0.00	$\pm$ 0.00	0.81	$\pm$ 0.13	-0.32	-0.65	0.88	0.81
4 Kologha	13	4	5	0.00	$\pm$ 0.00	0.42	$\pm$ 0.16	-1.86*	-1.08	0.00*	1.00
5 Kubusi	3	1	1	0.00	$\pm$ 0.00	0.67	$\pm$ 0.31	0.00	0.20	0.55	0.94

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**Table 6:** Pairwise  $F_{ST}$  values among sampled localities of *Anhydrophryne rattrayi*. The \* indicates  $p < 0.05$ .

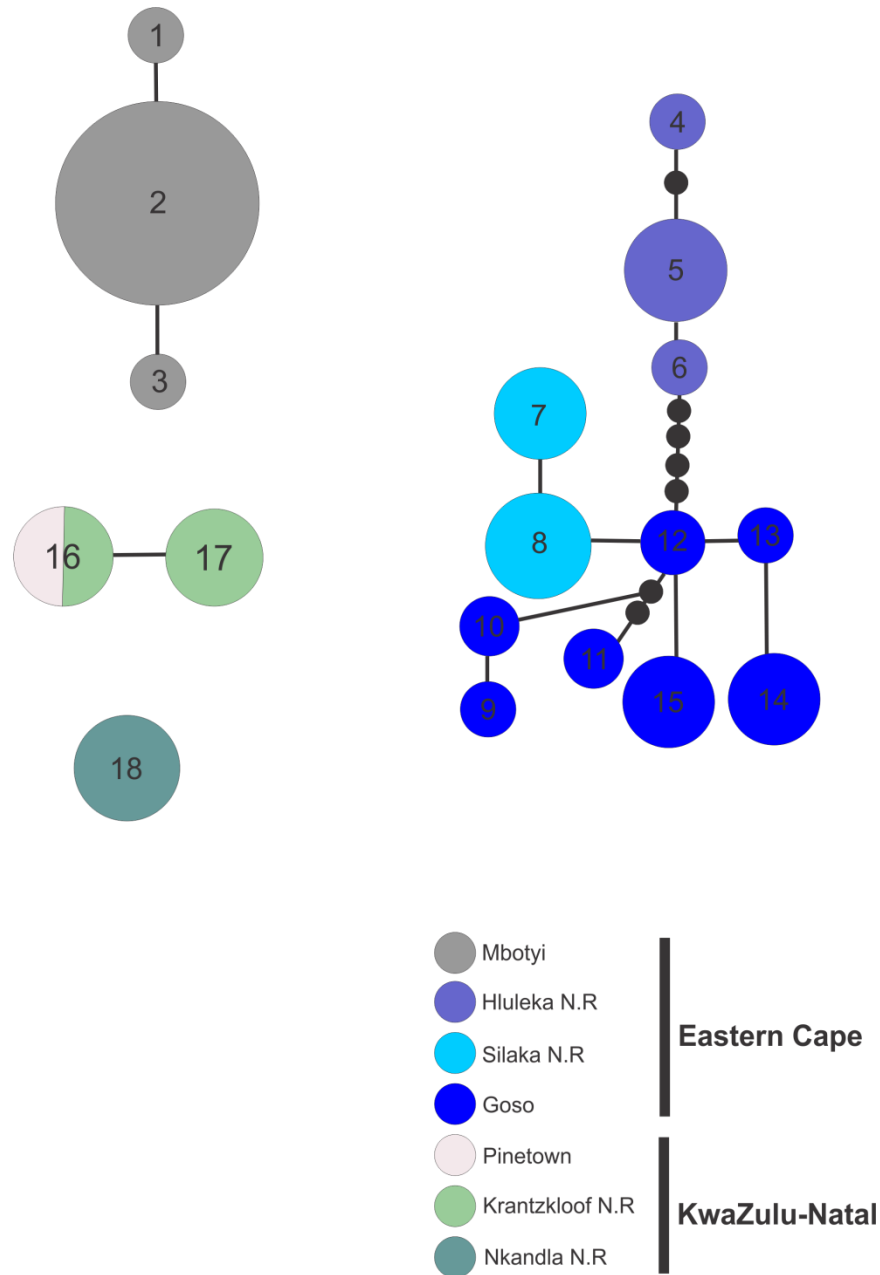
	<b>Isidenge</b>	<b>Katberg</b>	<b>Hogsback</b>	<b>Kologha</b>	<b>Kubusi</b>
<b>Isidenge</b>	-				
<b>Katberg</b>	0.96*	-			
<b>Hogsback</b>	0.94*	0.97*	-		
<b>Kologha</b>	0.94*	0.98*	0.97*	-	
<b>Kubusi</b>	0.92*	0.98*	0.96*	0.30	-

### 3.3.2 *Arthroleptis wahlbergii*

The TCS analyses retrieved a total of 15 Cyt *b* haplotypes for the 38 specimens sequenced from the Eastern Cape Province forests whereas three haplotypes for the eight specimens sequenced from the KwaZulu-Natal Province forests were observed (Fig. 10). From the Eastern Cape Province, haplotypes from Goso, Silaka N.R and Hluleka N.R were connected into a single network. However, Mbotyi formed a separate and non-connected haplotype from the remaining Eastern Cape Province haplotypes. For the three localities from the KZN Province forests, TCS analyses retrieved two separate haplotype groups; the single Pinetown specimen was connected to the specimens from Krantzkloof N.R, sharing one of the two haplotypes whereas specimens from Nkandla N.R formed a single and unconnected haplotype (Fig. 10, Table 7). The number of polymorphic sites was low, ranging between one and seven within the Eastern Cape Province forests. Both nucleotide diversity and gene/haplotype diversity were generally low (Table 8), except for Goso. Among the Eastern Cape Province populations of *A. wahlbergii*, 93.22% of the variation was present between sampled localities (d.f. =3, sum of squares =241.05,  $V_a = 8.65$ ), whereas 6.78% of the variation was present within sampled populations (d.f. =34, sum of squares =21.40,  $V_b = 0.63$ ). Highly significant pairwise  $F_{ST}$  values (Table 9) were observed across all the sampled localities from the coastal forests of the Eastern Cape Province. Three localities within the Eastern Cape forests had negative and non-significant  $F_u$ 's  $F_s$  as well as low and negative Tajima's  $D$  indices (Table 8). Exceptions were the populations from Silaka N.R which had a positive (0.86) although statistically non-significant  $F_u$ 's  $F_s$  and Hluleka N.R which was significant for Tajima's  $D$   $p = 0.08$ . Low and non-significant raggedness and SSD values ( $p > 0.05$ ) in *A. wahlbergii* (Table 8) suggest that the species might not have undergone a sudden population expansion in history.



Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.



**Figure 10:** Haplotype networks of *Arthroleptis wahlbergii* (46 sequenced specimens) based on 543bp of the Cyt *b* locus. Each circle represents one haplotype; number inside each haplotype represents haplotype name; size of circle is proportional to haplotype frequency and color represents population/ sample locality corresponding to Fig. 2. The partitions within the circles represent the proportion of each sampled population within a haplotype. Black dots represent putative haplotypes that were unsampled or are missing.

Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.

**Table 7:** Summary of the Cyt *b* haplotype frequencies between and within *Arthroleptis wahlbergii* sample localities (corresponds to Fig. 10).

Locality	Haplotypes																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
1 Goso									1	2	2	2	1	3	3				
2 Hluleka N.R				1	5	1													
3 Silaka N.R							3	4											
4 Mbotyi	1	10	1																
5 Krantzkloof N.R																1	2		
6 Pinetown																1			
7 Nkandla N.R																			4

Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.

**Table 8:** Summary list of the population parameters for each of the *Arthroleptis wahlbergii* Eastern Cape sampled localities. N is the number of samples per locality,  $N_h$  is the number of haplotypes and  $N_p$  is the number of polymorphic sites. The \* indicates  $p < 0.02$ ,  $< 0.10$ ,  $< 0.05$  for Fu's  $F_s$ , Tajima's  $D$  and mismatch distribution parameters SSD and  $r$ , respectively.

Locality	N	$N_h$	$N_p$	Nucleotide diversity ( $\pm$ SD)	Gene/haplotype diversity ( $\pm$ SD)	Tajima's $D$	Fu's $F_s$	Mismatch distribution	
								SSD $p$	$r p$
1 Goso	14	7	7	0.00 $\pm$ 0.00	0.90 $\pm$ 0.05	0.49	-1.41	0.47	0.63
2 Silaka N.R	7	2	1	0.00 $\pm$ 0.00	0.57 $\pm$ 0.12	1.34	0.86	0.37	0.24
3 Hluleka N.R	7	3	3	0.00 $\pm$ 0.00	0.52 $\pm$ 0.21	-1.36*	-0.24	0.79	0.94
4 Mbotyi	10	3	1	0.00 $\pm$ 0.00	0.20 $\pm$ 0.15	-1.11	-0.34	0.10	0.14

Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.

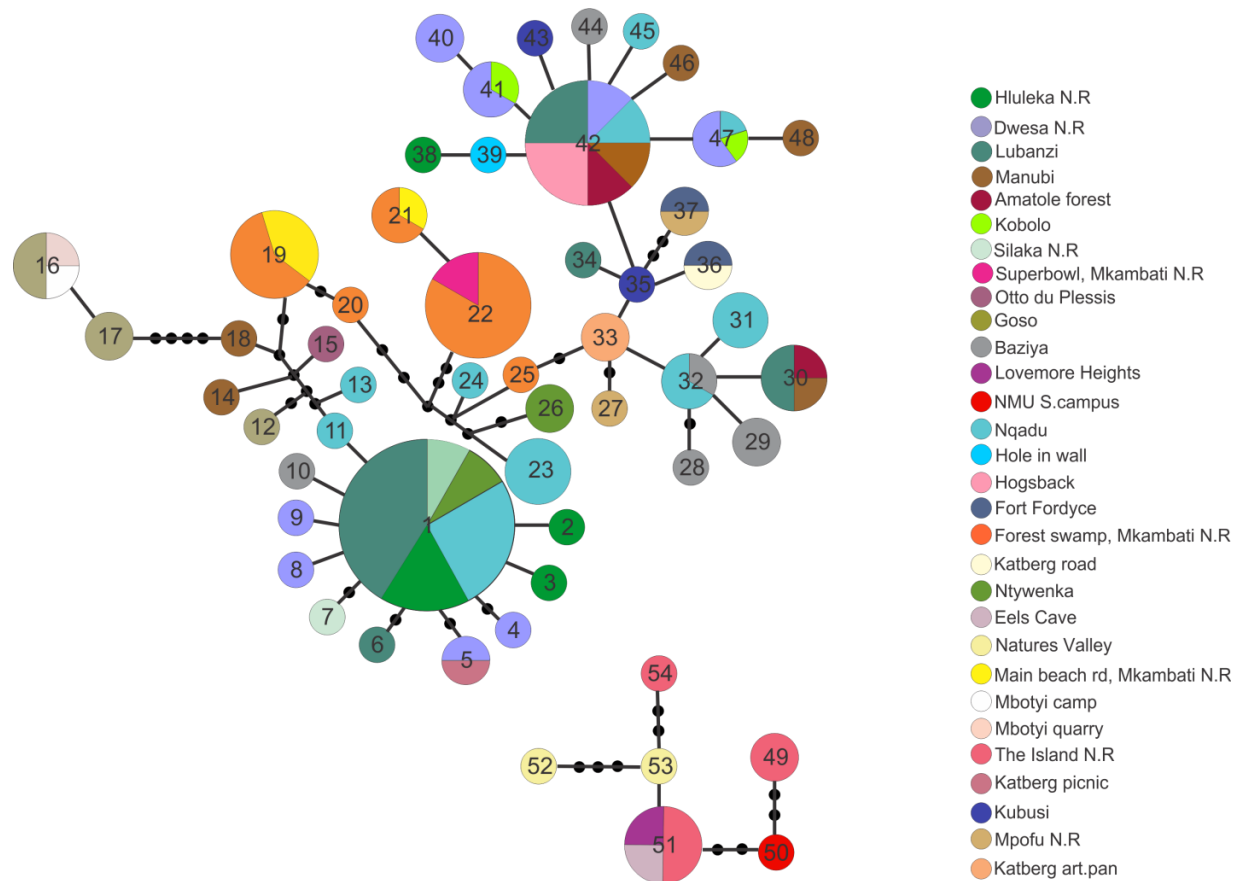
**Table 9:** Pairwise  $F_{ST}$  values among sampled localities of *Arthroleptis wahlbergii*. The \* indicates  $p < 0.05$ .

	<b>Goso</b>	<b>Silaka N.R</b>	<b>Hluleka N.R</b>	<b>Mbotyi</b>
<b>Goso</b>	-			
<b>Silaka N.R</b>	0.75*	-		
<b>Hluleka N.R</b>	0.47*	0.91*	-	
<b>Mbotyi</b>	0.95*	0.98*	0.99*	-

### 3.3.3 *Cacosternum nanum*

The TCS analyses retrieved a total of 54 Cyt *b* haplotypes for the 114 *C. nanum* specimens sequenced. Two haplotype networks were retrieved at 95% confidence level (Fig. 11). The first network comprised of 48 haplotypes from 25 localities with most sample localities sharing haplotypes (Fig. 11, Table 10) indicating high connectivity between populations, suggesting high levels of gene flow among populations. Specimens from Nqadu dominate the first network, albeit in different frequencies. The second network comprised specimens from Eels Cave, Lovemore Heights, NMU S.campus, The Island N.R and the Western Cape Province specimens from Natures Valley. Although the latter five sample localities are connected into a single framework, only specimens from The Island N.R, Eels Cave and Lovemore Heights shared a haplotype (Fig. 11, Table 10). The number of polymorphic sites and nucleotide diversity was low whereas gene/ haplotype diversity was high (Table 11). Among *C. nanum* populations, 45.66% of the variation was present between sampling localities (d.f. = 29, sum of squares =217.879,  $V_a = 1.94$ ), whereas 54.34% of the variation was present within sampling localities observed (d.f. =84, sum of squares =198.79,  $V_b = 2.31$ ). Significant pairwise  $F_{ST}$  are presented in Table 12. Fu's  $F_s$  was generally low (<1.00) although the Main beach road, Mkambati N.R population had a value of 3.30. Tajima's  $D$  indices were low and negative with a few moderate exceptions of the sample populations from Kubusi, The Island N.R, Nqadu, Lubanzi and Dwesa N.R (Table 11). All populations were statistically non-significant for Fu's  $F_s$  and Tajima's  $D$  except for Hluleka, significant for Tajima's  $D$  at  $p = 0.05$ . The mismatch distributions for the Cyt *b* locus for *C. nanum* were generally low and non-significant (Table 11) across populations suggesting that the species might not have undergone a sudden population expansion in history. However, a significant raggedness ( $p = 0.02$ ) and SSD values ( $p = 0.04, 0.01$ ) found in *C. nanum* populations from Lubanzi, Ntywenka and Main beach road, Mkambati N.R, respectively (Table 11) suggest that these three populations may have undergone a population expansion in history.

Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.



**Figure 11:** Haplotype networks of *Cacosternum nanum* (114 sequenced specimens) based on 531bp of the *Cyt b* locus. Each circle represents one haplotype; number inside each haplotype represents haplotype name; size of circle is proportional to haplotype frequency and color represents population/ sample locality corresponding to Fig. 3. The partitions within the circles represent the proportion of each sampled population within a haplotype. Black dots represent putative haplotypes that were unsampled or are missing.

Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.

**Table 10:** Summary of the Cyt *b* haplotype frequencies among *Cacosternum nanum* sample localities (corresponds to Fig. 11).

Locality	Haplotypes																										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1 The Island N.R																											
2 NMU S-campus																											
3 Eels Cave																											
4 Natures Valley																											
5 Hluleka N.R	2	1	1																								
6 Dwesa N.R				1	1			1	1																		
7 Lubanzi	5					1																					
8 Silaka N.R	1						1																				
9 Baziya										1																	
10 Nqadu	3										1		1										4	1			
11 Goso												1				2	2										
12 Manubi														1				1									
13 Forest Swamp, Mkambati N.R																			3	1	2	5			1		
14 Otto du Plessis Pass															1												
15 Ntywenka	1																									2	
16 Hole in wall																											
17 Katberg artificial pan																											
18 Mpofu N.R																											1
19 Kubusi																											
20 Katberg picnic					1																						
21 Katberg road																											
22 Amatole forest																											
23 Fort Fordyce																											
24 Mbotyi quarry																1											
25 Mbotyi campsite															1												
26 Main beach road, Mkambati N.R																			2		1						
27 Superbowl, Mkambati N.R																							1				
28 Kobolo																											
29 Hogsback																											
30 Lovemore Heights																											

Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.

**Table 10:** continues

Locality	Haplotypes																										
	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
1 The Island N.R																						2		2			1
2 NMU S.campus																							1				
3 Eels Cave																								1			
4 Natures Valley																										1	1
5 Hluleka N.R											1																
6 Dwesa N.R													2	2	1						3						
7 Lubanzi				2			1								2												
8 Silaka N.R																											
9 Baziya	1	2			1												1										
10 Nqadu				3	2										1			1		1							
11 Goso																											
12 Manubi				1											1				1		1						
13 Forest Swamp, Mkambati N.R																											
14 Otto du Plessis Pass																											
15 Ntywenka																											
16 Hole in wall												1															
17 Katberg artificial pan							2																				
18 Mpofu N.R											1																
19 Kubusi									1							1											
20 Katberg picnic																											
21 Katberg road										1																	
22 Amatole forest				1											1												
23 Fort Fordyce										1	1																
24 Mbotyi quarry																											
25 Mbotyi campsite																											
26 Main beach road, Mkambati N.R																											
27 Superbowl, Mkambati N.R																											
28 Kobolo														1							1						
29 Hogsback															2												
30 Lovemore Heights																									1		



Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.

**Table 11:** Summary list of the population parameters for each of the *Cacosternum nanum* sampled localities. N is the number of samples per locality, Nh is the number of haplotypes and Np is the number of polymorphic sites. A '-' indicates a computational failure due to presence of one gene or mismatch variance too small for demographic estimates to be computed. The \* indicates  $p < 0.02$ ,  $< 0.10$ ,  $< 0.05$  for Fu's  $F_s$ , Tajima's  $D$  and mismatch distribution parameters SSD and  $r$ , respectively.

	Locality	N	Nh	Np	Nucleotide diversity ( $\pm$ SD)		Gene/haplotype diversity ( $\pm$ SD)		Tajima's $D$	Fu's $F_s$	Mismatch distribution	
											SSD $p$	$r p$
1	The Island N.R	5	3	9	0.01	$\pm$ 0.01	0.80	$\pm$ 0.16	0.79	2.23	0.17	0.20
2	NMU S.campus	1	1	0	0.00	$\pm$ 0.00	1.00	$\pm$ 0.00	0.00	0.00	-	-
3	Eels Cave	1	1	0	0.00	$\pm$ 0.00	1.00	$\pm$ 0.00	0.00	0.00	-	-
4	Natures Valley	2	2	4	0.01	$\pm$ 0.01	1.00	$\pm$ 0.50	0.00	1.39	-	-
5	Hluleka N.R	5	4	11	0.01	$\pm$ 0.01	0.90	$\pm$ 0.16	-1.20*	0.29	0.10	0.38
6	Dwesa N.R	12	8	16	0.01	$\pm$ 0.01	0.92	$\pm$ 0.58	0.24	-0.75	0.06	0.56
7	Lubanzi	11	5	12	0.01	$\pm$ 0.01	0.78	$\pm$ 0.11	0.63	1.71	0.10	0.02*
8	Silaka N.R	2	2	2	0.00	$\pm$ 0.00	1.00	$\pm$ 0.50	0.00	0.69	-	-
9	Baziya	6	5	12	0.01	$\pm$ 0.01	0.93	$\pm$ 0.12	-0.76	-0.50	0.94	0.99
10	Nqadu	18	10	18	0.01	$\pm$ 0.01	0.92	$\pm$ 0.04	0.64	-1.21	0.26	0.42
11	Goso	5	3	9	0.01	$\pm$ 0.01	0.80	$\pm$ 0.16	-0.86	1.77	0.26	0.48
12	Manubi	6	6	13	0.01	$\pm$ 0.01	1.00	$\pm$ 0.10	-0.24	-1.99	0.77	0.98
13	Forest Swamp, Mkambati N.R	12	5	10	0.01	$\pm$ 0.00	0.79	$\pm$ 0.09	1.10	1.67	0.17	0.49
14	Otto du Plessis Pass	1	1	0	0.00	$\pm$ 0.00	1.00	$\pm$ 0.00	0.00	0.00	-	-
15	Ntywenka	3	2	6	0.01	$\pm$ 0.01	0.67	$\pm$ 0.31	0.00	2.64	0.04*	0.60
16	Hole in wall	1	1	0	0.00	$\pm$ 0.00	1.00	$\pm$ 0.00	0.00	0.00	-	-
17	Katberg artificial pan	2	1	0	0.00	$\pm$ 0.00	0.00	$\pm$ 0.00	0.00	0.00	-	-
18	Mpofu N.R	2	2	6	0.01	$\pm$ 0.01	1.00	$\pm$ 0.50	0.00	1.79	-	-
19	Kubusi	2	2	2	0.00	$\pm$ 0.00	1.00	$\pm$ 0.50	0.00	0.69	-	-
20	Katberg picnic	1	1	0	0.00	$\pm$ 0.00	1.00	$\pm$ 0.00	0.00	0.00	-	-
21	Katberg road	1	1	0	0.00	$\pm$ 0.00	1.00	$\pm$ 0.00	0.00	0.00	-	-
22	Amatole forest	2	2	4	0.01	$\pm$ 0.01	1.00	$\pm$ 0.50	0.00	1.39	-	-
23	Fort Fordyce	2	2	4	0.01	$\pm$ 0.01	1.00	$\pm$ 0.50	0.00	1.39	-	-
24	Mbotyi quarry	1	1	0	0.00	$\pm$ 0.00	1.00	$\pm$ 0.00	0.00	0.00	-	-
25	Mbotyi campsite	1	1	0	0.00	$\pm$ 0.00	1.00	$\pm$ 0.00	0.00	0.00	-	-
26	Main beach road, Mkambati N.R	3	2	9	0.01	$\pm$ 0.01	0.67	$\pm$ 0.31	0.00	3.30	0.01*	0.54
27	Superbowl, Mkambati N.R	1	1	0	0.00	$\pm$ 0.00	1.00	$\pm$ 0.00	0.00	0.00	-	-
28	Kobolo	2	2	2	0.00	$\pm$ 0.00	1.00	$\pm$ 0.50	0.00	0.69	-	-
29	Hogsback	2	1	0	0.00	$\pm$ 0.00	0.00	$\pm$ 0.00	0.00	0.00	-	-
30	Lovemore Heights	1	1	0	0.00	$\pm$ 0.00	1.00	$\pm$ 0.00	0.00	0.00	-	-

Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.

**Table 12:** Pairwise  $F_{ST}$  values among sampled localities of *Cacosternum nanum*. The \* indicates  $p < 0.05$ .

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
1	-																			
2	-0.26	-																		
3	-0.50	1.00	-																	
4	0.89	0.33	-0.33	-																
5	0.72*	0.75	0.70	0.72	-															
6	0.68*	0.68	0.62	0.66*	0.18	-														
7	0.71*	0.73	0.67	0.70*	-0.02	0.09	-													
8	0.76*	0.89	0.87	0.81	-0.12	0.30	0.05	-												
9	0.72*	0.74	0.68	0.71*	0.28	0.14	0.09	0.41	-											
10	0.68*	0.69*	0.63*	0.68*	0.12	0.13*	0.03	0.21	0.09	-										
11	0.78*	0.82	0.80	0.80	0.58*	0.52*	0.55*	0.65*	0.62*	0.52*	-									
12	0.68*	0.68	0.61	0.66	0.24*	-0.01	0.08	0.36*	0.04	0.04	0.51*	-								
13	0.78*	0.80	0.77	0.78	0.33*	0.44*	0.36*	0.35	0.51*	0.36*	0.48*	0.45*	-							
14	0.72	1.00	1.00*	0.75	0.00	0.11	-0.03	0.50	0.21	-0.15	0.50	-0.03	0.20	-						
15	0.73*	0.76	0.71	0.76	0.25	0.37	0.27	0.35	0.44*	0.18	0.61*	0.34*	0.42*	0.08	-					
16	0.70	1.00	1.00	0.73	0.37	-0.22	0.21	0.78	0.11	0.17	0.65	-0.26	0.59	1.00	0.57	-				
17	0.75	1.00	1.00	0.86	0.39	0.04	0.09	0.83	-0.10	0.01	0.70*	-0.11	0.52*	1.00	0.59	1.00	-			
18	0.71	0.68	0.63	0.71	0.38	0.14*	0.24*	0.56	0.12	0.20	0.63	0.04	0.56*	0.14	0.49	-0.20	0.00	-		
19	0.74	0.88	0.86	0.80	0.43	-0.07	0.20	0.75	0.11	0.17	0.66*	-0.13	0.59*	0.67	0.61	0.00	0.50	0.00	-	
20	0.73	1.00	1.00	0.76	-0.05	0.26	0.13	0.33	0.41	0.22	0.64	0.34	0.38	1.00	0.33	1.00	1.00	0.40	0.78	
21	0.72	1.00	1.00	0.75	0.35	-0.14	0.11	0.75	-0.02	0.06	0.62	-0.26	0.55	1.00	0.52	1.00	1.00	-0.50	0.00	
22	0.71*	0.76	0.71	0.73	0.37	-0.05	0.10	0.60	-0.20	0.07	0.64	-0.24	0.55*	0.33	0.52	-0.33	0.00	-0.11	-0.20	
23	0.74	0.72	0.75	0.76	0.44	0.12	0.28	0.67	0.19	0.25*	0.65*	0.06	0.59*	0.43	0.57	0.00	0.33	-0.43	0.00	
24	0.77	1.00	1.00	0.81	0.60	0.52	0.57	0.82	0.63	0.54	-0.73	0.43	0.54	1.00	0.65	1.00	1.00	0.54	0.81	
25	0.77	1.00	1.00	0.81	0.60	0.52	0.57*	0.82	0.63	0.54	-0.73	0.43	0.54	1.00	0.65	1.00	1.00	0.54	0.81	
26	0.74*	0.72	0.67	0.73	0.30*	0.36*	0.32	0.32	0.44*	0.30*	0.33	0.33*	0.03	-0.13	0.33	0.42	0.48	0.42	0.51	
27	0.76	1.00	1.00	0.79	0.24	0.35	0.27	0.60	0.46	0.27	0.54	0.34	-0.53	1.00	0.40	1.00	1.00	0.40	0.78	
28	0.74*	0.88	0.86	0.80	0.49	0.13	0.31*	0.78	0.25	0.27	0.69*	-0.05	0.62*	0.71	0.65	0.00	0.67	0.20	0.00	
29	0.75*	1.00	1.00	0.86	0.49	-0.10	0.26	0.88	0.20	0.22	0.71	-0.11	0.62*	1.00	0.69	1.00	1.00	0.25	0.00	
30	-0.50	1.00	1.00	-0.33	0.70	0.62	0.67	0.87	0.68	0.63	0.80	0.61	0.77	1.00	0.71	1.00	1.00	0.63	-0.86	

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**Table 12:** continues

	20	21	22	23	24	25	26	27	28	29	30
20	-										
21	1.00	-									
22	0.56	-0.33	-								
23	0.60	-1.00	0.00	-							
24	1.00	1.00	0.67	0.00	-						
25	1.00	1.00	0.67	0.00	0.00	-					
26	0.22	0.36	0.44	0.49	0.22	0.22	-				
27	1.00	1.00	0.56	0.60	1.00	1.00	-0.06	-			
28	0.80	0.33	0.00	0.25	0.83	0.83	0.54	0.80	-		
29	1.00	1.00	0.00	0.33	1.00	0.59	1.00	0.00	0.00	-	
30	1.00	1.00	0.71	0.75	1.00	1.00	0.67	1.00	0.86	1.00	-

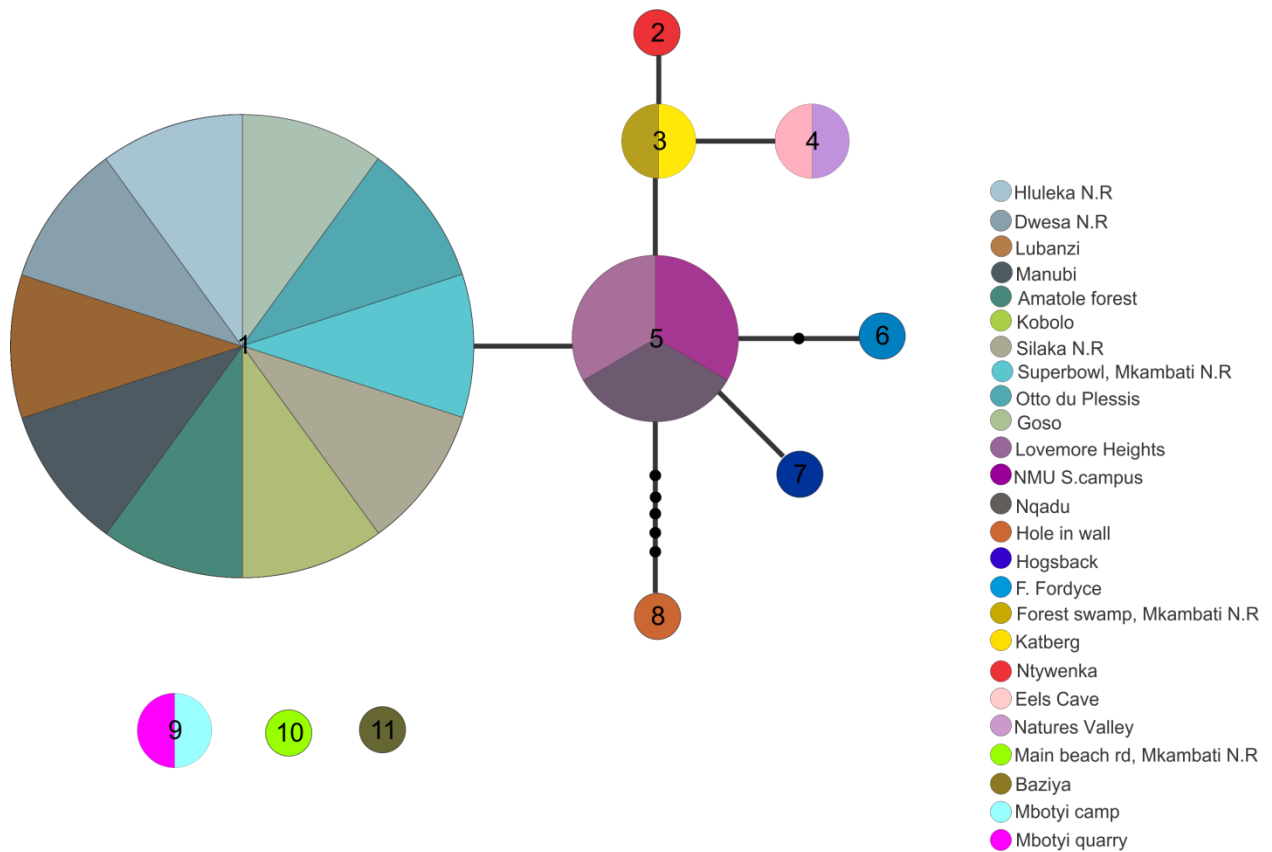
1= The Island N.R, 2= NMU S.campus, 3= Eels Cave, 4= Natures Valley, 5= Hluleka N.R, 6= Dwesa N.R, 7= Lubanzi, 8= Silaka N.R, 9= Baziya, 10= Nqadu, 11= Goso, 12= Manubi, 13= Forest Swamp, Mkambati N.R, 14= Otto du Plessis Pass, 15= Ntywenka, 16= Hole in wall, 17= Katberg artificial pan, 18= Mpofu N.R, 19= Kubusi, 20= Katberg picnic, 21= Katberg road, 22= Amatole forest, 23= Fort Fordyce, 24= Mbotyi quarry, 25= Mbotyi campsite, 26= Main beach road, Mkambati N.R, 27= Superbowl, Mkambati N.R, 28= Kobolo, 29= Hogsback, 30= Lovemore Heights.

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### 3.4 Nuclear markers (Recombination Activation Gene 1 and Rhodopsin)

The use of nuclear markers in genetics is known to reveal resolution further back in time (older divergence events/lineages) than those inferred from mtDNA. Using nuclear loci RAG1 and Rhod, the phylogenetic analyses did not reveal any distinct clades (topology not shown) for any of the three species. The TCS analyses retrieved two Rhod haplotypes (network not shown) for the five *Anhydrophryne rattrayi* specimens sequenced (one specimen sequenced per locality). Specimens from Hogsback, Katberg, Kologha and Kubusi shared one haplotype whereas specimens from Isidenge formed a separate and unconnected haplotype. In contrast, for the RAG1 locus, TCS analyses retrieved three haplotypes (network not shown), Hogsback and Katberg shared a haplotype, Kologha and Kubusi shared another whereas Isidenge did not share a haplotype with any localities. For the seven *Arthroleptis wahlbergii* specimens sequenced from both the Eastern Cape coastal forests and the KZN forests, TCS analyses retrieved a single RAG1 network (not shown) comprising of three connected haplotypes. The four localities from the coastal forests of the Eastern Cape Province shared a haplotype connected to four unsampled haplotypes between the single Nkandla N.R haplotype and the haplotype shared between Pinetown and Krantzklouf N.R. The nuclear Rhod TCS network for *Cacosternum nanum* indicated levels of population connectivity (Fig. 12). From the 25 specimens sequenced, the TCS analysis retrieved a total of 11 Rhod haplotypes (Fig. 12) with 21 of the localities connecting into one framework and sharing haplotypes (Fig. 12, Table 13) whereas the remaining four localities (Mbotyi quarry, Mbotyi camp, Main Beach road, Mkambati N.R and Baziya) formed three separate and unconnected haplotypes, the two Mbotyi localities shared a haplotype.

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**Figure 12:** Haplotype networks of *Cacosternum nanum* from 25 localities (one specimen sequenced per each locality), based on 292bp of the Rhodopsin locus. Each circle represents one haplotype; number inside of haplotype represents haplotype name; size of circle is proportional to haplotype frequency and color represents population/ sample locality corresponding to Fig. 3. The partitions within the circles represent the proportion of each sampled population within a haplotype. Black dots represent putative haplotypes that were unsampled or are missing.

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**Table 13:** Summary of the Rhodopsin haplotype frequencies among *Cacosternum nanum* sample localities (corresponds to Fig. 12).

	Locality	Haplotypes										
		1	2	3	4	5	6	7	8	9	10	11
1	Hluleka N.R	1										
2	Dwesa N.R	1										
3	Lubanzi	1										
4	Manubi	1										
5	Amatole forest	1										
6	Kobolo	1										
7	Silaka N.R	1										
8	Superbowl, Mkambati N.R	1										
9	Otto du Plessis Pass	1										
10	Goso	1										
11	Lovemore Heights					1						
12	NMU S.campus					1						
13	Nqadu					1						
14	Hole in wall								1			
15	Hogsback							1				
16	Fort Fordyce						1					
17	Forest Swamp, Mkambati N.R			1								
18	Katberg road			1								
19	Ntywenka		1									
20	Eels Cave				1							
21	Natures Valley				1							
22	Main beach road, Mkambati N.R									1		
23	Baziya											1
24	Mbotyi campsite								1			
25	Mbotyi quarry								1			

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# **CHAPTER FOUR DISCUSSION**

## Chapter 4

### Discussion

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#### 4. Discussion

##### 4.1 General introduction

All three, anuran species, *Anhydrophryne rattrayi*, *Arthroleptis wahlbergi* and *Cacosternum nanum* were monophyletic, with the former two forest specialist species exhibiting limited to no maternal connections between sample localities, had marked  $F_{ST}$  values and were highly divergent for the Cyt *b* locus. In contrast, the latter generalist species shared haplotypes, comparatively lower  $F_{ST}$  values and less divergent values for the Cyt *b* locus. The two forest specialists, *Anhydrophryne rattrayi* and *Arthroleptis wahlbergi* exhibited deeper Plio/Pleistocene divergences while the generalist species, *Cacosternum nanum* exhibited divergences associated with the Quaternary Period, particularly, the Pleistocene epoch. Climatic shifts during glacial and interglacial cycles of the Late Pliocene to Pleistocene may have shaped the evolution and diversification of the two forest dependent anuran taxa in the Eastern Cape Province of South Africa. These results are consistent with the earlier prediction that specialist anuran taxa exhibit strong genetic structuring as they depend on specific habitats unlike generalists that make use of various habitats and are less severely impacted by habitat fragmentation and degradation.

Ecologically sensitive taxa that survived paleoclimatic extinction filtering processes are known to exhibit greater persistence and agility (Balmford, 1996; Lawes *et al.*, 2000) and are less susceptible to further forest fragmentation or deforestation (Balmford, 1996; Danielsen, 1997). Seemingly, species habitat preference and specificity depend on the extent of environmental fluctuations and stressors forests underwent in the past (Balmford, 1996; Attum *et al.*, 2006; Tolley *et al.*, 2006). In addition, Fjeldsa° *et al.* (2005) and Schneider and Williams (2005) suggest that forest dependant taxa that were affected by the Pleistocene climatic conditions were more adaptable to various forest types than those affected by preceding epochs. Therefore, the differences in ecological mechanisms between the responses of the two specialists and the generalist during periods of dramatic climatic cycles may be partially responsible for the observed differences in their phylogeographic patterning. In this respect, the present study provides corroborative evidence in support of all three of the alternative hypotheses proposed in the thesis introduction.



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## 4.2 *Anhydrophryne rattrayi*

### 4.2.1 Phylogenetics

Within *Anhydrophryne rattrayi*, two divergent clades were observed and were estimated to have diversified during the Plio-Pleistocene epoch. Clade 1, comprising specimens from Katberg sister to Hogsback and diverged between the Late Pliocene and the onset of Pleistocene. Clade 2 comprising specimens from Isidenge, sister to Kologha and Kubusi diverged during the Early Pleistocene. These results corroborate the influence of Plio-Pleistocene climates on the evolution as observed from other African fauna (e.g. *bovids*: Vrba, 1995; *rodents*: Matthee and Flemming, 2002; *lizards*: Daniels *et al.*, 2004; de Menocal, 2004; *crabs*: Daniels *et al.*, 2006; *chameleons*: Tolley *et al.*, 2008). There is a paucity of information on the paleoclimatic variability in southern Africa during this period (de Menocal, 2004; Dupont *et al.*, 2013). However, it is suggested that the increase in intensity of the Benguela current between the Late Pliocene and onset of the glaciation cycles of the Pleistocene caused a gradual decrease in sea surface temperatures (Marlow *et al.*, 2000). Subsequently, the prolonged long-term cooling altered rainfall patterns in southern Africa (Lisiecki and Raymo, 2005) which encouraged grassland expansion and reduced forests (de Menocal, 2004; Hopley *et al.*, 2007). The increase in aridity may have further accelerated fragmentation of Afromontane forests (Mucina and Geldenhuys, 2006), more so, within the already isolated and highly elevated forests of the Amatole Mountains in South Africa. The Afromontane forest patches in this region generally occur at higher altitudes, restricting forest dependant taxa such as *Anhydrophryne rattrayi* to habitat refugia. These findings are consistent with the Plio-Pleistocene Forest Refuge (PFR) hypothesis postulated by Haffer (1969) who suggested that the contraction and expansion of forests in the African tropics during the cool and arid climatic periods led to allopatric speciation.

The landscape in the Amatole Mountains of South Africa, is characterised by high peaked mountainous ranges that exceed 1100m above sea level (a.s.l) (Burger, 2004; Dawood and Stam, 2006), separated by large expanses of dry corridors and deeply incised valleys (Burger, 2004). Among the five sample localities, Hogsback and Katberg forests represent habitats with peaks >1200m a.s.l (Burger, 2004) of Afromontane (mistbelt) forests whereas Kologha, Kubusi and Isidenge forests are lower lying and occur as isolated patches of indigenous forests among exotic Pine plantations. The two genetic lineages recovered within *A. rattrayi* (clades 1 and 2) conform to this landscape structuring. The geographical landscape patterning

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within clade 1 (see insert map: Fig. 4) indicates that Katberg and Hogsback represent high altitude and isolated populations with no direct zones of contact or “stepping stones”. It is unlikely that these two populations are currently connected, but rather that this relationship reflects an ancient (see Fig.5) connection between these forests. Kologha and Kubusi forests are adjacent to each other hence the genetic structure observed between those populations is plausible. Isidenge forest falls in the same forest belt as Kologha and Kubusi, although separated by about 14km, a dam, a major road (R352) and lies further to the south, potentially impeding gene flow between these populations.

#### 4.2.2 Population structure

Small bodied specialist species occurring within isolated and fragmented habitats are expected to exhibit low genetic diversity due to effects of inbreeding and genetic drift which results in small population sizes (Frankham, 1995; Daniels *et al.*, 2016). Thus, based on the occurrence of *A. rattrayi* in isolated Afromontane forest fragments, the present study anticipated low gene and nucleotide diversity within *A. rattrayi* populations. Instead, gene diversity was moderately high (close to 1), and nucleotide diversity was low within *A. rattrayi* populations implying that there could be essential factors increasing gene diversity within *A. rattrayi* populations within the isolated and fragmented Amatole Mountains. The low dispersal ability of frogs (Avice, 2000; Smith and Green, 2005) coupled with their dependence on moisture, small size, high philopatry to natal sites (Duellman and Trueb, 1986; Blaustein *et al.*, 1994; Smith and Green, 2005), the direct development trait within *Anhydrophryne* (Burger, 2004; Dawood and Stam, 2006) and the fragmented habitats in which *A. rattrayi* occur may be factors responsible for the population genetic structure observed among populations. Interestingly, during fieldwork, previously toe-clipped frogs (n =4) were recaptured collectively in Kologha and Isidenge six months from the initial capture and release, confirming the limited dispersal abilities and high philopatry to natal sites of terrestrial frogs, concordant to findings of Smith and Green (2006) on wood frogs and Ringler *et al.* (2009) on brilliant-thighed poison frogs. Thus, philopatry may be regarded as a good option for *A. rattrayi* given that it relies on moist leaf litter for reproduction and evade desiccation.

Additionally, it has been also been documented that, over time genetically isolated populations will exhibit marked genetic diversity as they are able to adapt in a stable environment and persist, in refugia (e.g. Medrano and Herrera, 2008; Aleksić and Geburek,

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2010). Based on Fu's  $F_s$ , there was no evidence of selection for all populations, suggesting that *A. rattrayi* populations are neutrally evolving, corroborating the marked population structure and high gene diversity between populations. Furthermore, the absence of shared haplotypes among all populations except between Kologha and Kubusi, suggests that physical barriers such as roads (Hitchings and Beebee, 1997), rivers (Lampert *et al.*, 2003) and mountain ranges (Funk *et al.*, 2005), large geographical distance as well as environmental conditions can impact connectivity and gene flow. Collectively, these results shed light on the importance of maintaining and conserving forest patches for the survival of these vulnerable species.

#### **4.2.3 Do the two clades in *Anhydrophryne rattrayi* represent distinct species?**

The delimitation of species has been a contested and argued about issue in systematics (de Queiroz, 2007a). In most frogs, 16S rRNA is widely used in taxonomy, to corroborate other approaches in the delimitation of species (see Dawood and Stam, 2006; Blackburn, 2008b; Zimkus, 2009; Rödel *et al.*, 2012; Zimkus and Gvozdík, 2013). However, the utility of the mitochondrial DNA loci, Cyt *b*, in resolving divergences has been suggested among a wide range of vertebrate taxa (e.g. mammals: Irwin *et al.*, 1991; frogs: Graybeal, 1993; tortoises: Lamb and Lydeard, 1994; birds: Moore and de Filippis, 1997; fishes: Farias *et al.*, 2001). Kartavtsev (2011) identified and categorised divergence at Cyt *b* and CO1 loci on several taxonomic ranks. He suggested that for Cyt *b*, a genetic distance of  $1.38\% \pm 0.30\%$  was sufficient to define populations within a species and assigned  $5.10\% \pm 0.91\%$  for subspecies, semi-species or sibling species. The sequence divergence for the Cyt *b* locus between *Anhydrophryne rattrayi* populations in the present study ranged between 0.20% and 6.70%. These findings are concordant with those of other amphibian populations (e.g. the orange-bellied newt: Tan and Wake, 1995; the red-legged frog: Shaffer *et al.*, 2004; Barrio's frog: Méndez *et al.*, 2006). Between the two genetically distinct clades, a 5.30% sequence divergence was observed. Thus, applying the cut-off suggested by Kartavtsev (2011) would imply that *A. rattrayi* clades potentially represent sibling or subspecies (see Mayr, 1942; Patten, 2015). However, this approach too, should be taken with caution. Several studies (e.g. Austin, 1952; Patten and Unitt, 2002; Haig *et al.*, 2006) suggest that allocating a geographical population as a subspecies is not feasible unless three quarters (75%) or more of that population can be morphologically identified as similar. Genetic distinctiveness alone cannot conclusively delimit the taxonomic standing of taxa as both phenotypic and genotypic

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variation should be assessed (de Queiroz, 2007a; Mousseau and Sikes, 2011; Patten, 2015). The present study cannot define the taxonomic standing of *A. rattrayi* clades within the Amatole Mountains, suggesting additional data be collected. However, the current IUCN listing of the species (VU), the marked mtDNA genetic variation and haplotype isolation between populations, absence of nuDNA genetic isolation and the diversification time estimates of populations, point towards a possibility of incipient allopatric speciation within *A. rattrayi* (e.g. see Crisp and Cook, 2012). Overall, the inclusion of other data such as morphological assessments, bioacoustics, and landscape genetic approaches which are known to identify possible species isolation mechanisms in frogs are suggested for future work (Stuart *et al.*, 2006; Feinberg *et al.*, 2014; Priti *et al.*, 2016).

### **4.3 *Arthroleptis wahlbergii***

#### **4.3.1 Phylogenetics**

The present study revealed two geographically structured and genetically highly supported clades from the Eastern Cape Province and one clade from the KwaZulu-Natal Province, albeit with high levels of substructure. The tree topology presented herein along with the divergence time estimates revealed KZN clade (clade 1) as basal to the Eastern Cape clade, while within the Eastern Cape clade, Mbotyi (clade 2) was sister to Hluleka, sister to Silaka and Goso (clade 3). The KZN clade diverged during the mid-Pliocene (mean: 3.92 mya) whereas the Eastern Cape clade diverged much earlier during the Late Miocene (mean: 5.76 mya). Given the difference in divergence times between the Eastern Cape clade and the KZN clade, it is plausible that the former clade may represent a coastal forest invasion which diverged during climatic perturbations of the Late Miocene epoch whereas the latter clade may represent a mainland invasion, diversifying much later with the increase in aridity causing regression of forests during the Pliocene. Interestingly, within the Eastern Cape clade, populations diverged much later during the Pleistocene. Clade 2, comprising specimens from Mbotyi diverged during the Late Pleistocene (mean: 0.12 mya), and clade 3 diversified sometime during the mid-Pleistocene (mean: 1.39 mya). These results indicate an increase in number of lineages during the Plio-Pleistocene and suggest that the interchange between glacial and interglacial cycles of this period which resulted in the intensification of the cool and arid conditions (Cerling, 1992; Lisiecki and Raymo, 2005) potentially further fragmented forests and isolated populations of *A. wahlbergii*. Thus, divergence of the Eastern Cape clades of *A. wahlbergii* seems to be mainly shaped by climatic events of the Pleistocene

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and conservatively, aligns with the PFR species diversification hypothesis (Haffer, 1969; de Menocal, 2004).

Additionally, despite being in close geographic proximity to Goso, Mbotyi formed a distinct clade with no zones of contact with the rest of the *A. wahlbergii* populations from the Eastern Cape clade. This may be due to the differences in slope and elevation between the two forests, which may subsequently influence environmental conditions. Mbotyi forest lies on the windward slope which provides a humid environment due to its proximity to the sea whereas Goso lies on the leeward slope with drier environmental conditions (rain shadow effect: Roe, 2005; Neumann and Bamford, 2015; Peterson, 2017). For example, Siler *et al.* (2013) highlights the strong variability in precipitation between the west and east slopes of the Washington Cascade Mountains in the United States of America, the former being wetter by at least 31%. The same influence of orographic gradient such as slope and elevation on gene flow and allopatric speciation has been observed in other frog studies (Funk *et al.*, 2005; Lowe *et al.*, 2006; Giordano *et al.*, 2007; Igawa *et al.*, 2012). Thus, the shifting climatic zones may have led the Goso and Mbotyi populations to adapt to contrasting environments leading to ecological isolation (see Coyne and Orr, 2004; Rundle and Nosil, 2005; Phillimore and Price, 2009; Schluter, 2009) resulting in restricted gene flow (Craw *et al.*, 2008; Haffer, 2008) and possible reproductive incompatibility (reproductive isolation). Interestingly, a recent study by Daniels (2017) which aimed at examining sympatric colour morphs within crabs (*Potamonateus*) revealed that two sympatric crabs represented two distinct taxa, with the new discovery occurring in Mbotyi forest (*P. mhlophe*), closely related to a species known from KZN forests (e.g. *P. dentatus*) and not to the sympatric *P. sidneyi* species. This suggests several scenarios; firstly, that there may be unprecedented factors within Mbotyi forest that may be responsible for cladogenesis among mobility restricted taxa and secondly, reasons for cladogenesis within Mbotyi may vary depending on taxa as within the present study.

Further, given the ecology of *A. wahlbergii* (terrestrial, forest dependent, direct developing, small bodied frog), contemporary landscape patterning of habitats, geographical distance between populations, the overall forest distribution within the Eastern Cape Province, it is highly unlikely that *A. wahlbergii* populations from Hluleka N.R, Silaka N.R and Goso can disperse between each other. Thus, a historical connection of these forests is a more plausible explanation. In Mexico, a study by Streicher *et al.* (2011) on a forest dependant frog taxon suggested that secondary climatic shifts within major climatic events may be responsible for

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the historical connection exhibited by populations in isolated forest patches. During cooler cycles, forests may have occurred at lower elevations, allowing dispersal and gene flow between populations that are restricted to higher elevations during warmer conditions (e.g. Savage, 2002).

#### **4.3.2 Population structure**

Forest specialists are expected to exhibit low genetic diversity due to small isolated populations being more susceptible to repeated bottlenecks caused by reductions in effective population size, genetic drift and inbreeding (Franzén and Nilsson, 2010; Li *et al.*, 2014). However, the population genetic structure of *A. wahlbergii* paints a complex picture. The mtDNA Cyt *b* marker revealed low nucleotide diversity and moderately high genetic diversity, indices that typically reflect a sudden population expansion in history (Avise, 2000) but Fu's  $F_s$  indices for all populations were not consistent with population expansion. Instead, neutrality test indices point towards no evidence of selection, indicating a balanced selection. These findings are consistent with the marked genetic variation between populations and the absence of shared haplotypes among the Eastern Cape Province clades, suggesting that gene flow is limited, although specimens from clade 3 indicate a historical connection. Delimiting recent population bottlenecks from effects of expansions have been known to be difficult (Tajima, 1989a; Tajima, 1989b; Ramírez-Soriano *et al.*, 2008). Thus, the inconclusive demographic history parameters in the present study could be counteracted by increasing sample size and factoring in the possibility of recombination (Ramírez-Soriano *et al.*, 2008).

#### **4.3.3 Possible cryptic species within a coastal forest endemic frog species?**

To determine the presence of a separate species, several factors are considered such as distinguishable morphological features, monophyly and reproductive isolation (de Queiroz, 2007a). Essentially, genetic variation leads to genetic incompatibility between populations, reproductive isolation corroborated by the lack of gene flow (absence of shared haplotypes) and subsequently inference of a separate species (Ferguson, 2002). Sufficient genetic divergence between species should be warranted (Johns and Avise, 1998). Thus, determining the exact cut-off value for sequence divergence proves difficult given the differences in loci used (Kartavtsev, 2011). For example, Kartavtsev (2011) tested two mtDNA loci on vertebrate and invertebrate taxa and discovered that sequence divergence increased with an increase in taxonomic rank level (from population to order). He suggested a cut off value of

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5.10%  $\pm$  0.91% for populations within a species and 10.31%  $\pm$ 0.93% for species within a genus using the Cyt *b* locus. The uncorrected sequence divergence for the Cyt *b* loci between the Eastern Cape clades and the KZN clade of *A. wahlbergii* was high, ranging between 11.70% and 12.20%. Interestingly, the sequence divergence between the two geographically close populations, Goso and Mbotyi within the Eastern Cape Province, lay between 5.90% and 6.70%, the highest difference among all four populations. Therefore, applying the cut-offs suggested by Kartavtsev (2011) warrants the KZN and Eastern Cape Province clades as separate species and the Mbotyi population as a sub-species or cryptic species within the Eastern Cape Province species. Several population level studies on other amphibian taxa have retrieved similar divergences indicating either cryptic species complexes or sub-species, respectively using the Cyt *b* locus to corroborate other lines of evidence in species delimitation (e.g. gray frogs: 0.18%-3.40% Ptacek *et al.*, 1994; leaf litter frog: 12.00%-15.00% Elmer *et al.*, 2007; salamanders: 12%-14% Vences and Wake, 2007; tungara frogs: 7.40% Pröhl *et al.*, 2010). However, analyses of the Cyt *b* locus on various taxa yield variable sequence divergences and taxonomic implications such that the reliability of using sequence divergence alone becomes problematic. For example, in Peruvian poison frogs (see Roberts *et al.*, 2006), a sequence divergence of 7.10% between clades was comparable to those between species of *Epipedobates* (7.90%-8.00%). Thus, multiple lines of evidence are suggested for future work.

#### **4.4 *Cacosternum nanum***

##### **4.4.1 Phylogeography**

In the *C. nanum* mtDNA phylogeny, specimens from the Island N.R, Eels Cave, Lovemore Heights, NMU S.campus and Natures Valley formed a highly supported clade whereas the rest of the specimens from the remaining 25 localities were highly unresolved. The former five localities represent the southernmost distribution of *C. nanum* from the study with a gap of over 250km between them and the closest locality from the northern distribution, creating a north/south division for *C. nanum*. An obscure relationship is revealed between populations from clade 2, representing the northern distribution of *C. nanum*. *Cacosternum nanum* is a widespread generalist species that is dependent on water throughout its life stages (Scott, 2004). Thus, a climate induced barrier is a plausible explanation for the differences observed in the genetic and geographical structure between clades. The northern clade of *C. nanum* (clade 2) experiences summer rainfall whereas the southern clade (clade 1) experiences

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winter rainfall with an aseasonal zone in-between the two clades. Interestingly, the north/south separation coincides with the Bedford gap described by Lawes (1990). The Bedford gap is characterised by succulent desert shrubs along a corridor of dry river valleys (Lubke *et al.*, 1988), whose semi-arid to arid conditions have been dated back to the onset of the Late Pleistocene (Geldenhuys, 1989). Divergence times estimated using coalescent approaches within the present study occur within the Pleistocene suggesting that this generalist species was exposed to intense cooling and arid climates of those times, but persistence would have been aided by its ability to actively disperse and interbreed and a reproductive mode that allows for spawning of eggs and a high metamorphosis rate which increases persistence of populations. In addition, the two lineages might have evolved independently of each other and maintained large effective population sizes, invoking the possibility of the influence of source and sink habitats (Pulliam, 1988) in maintaining populations of *C. nanum*. Given its high dispersal ability, immigrants from other local populations are likely able to disperse and reproduce, increasing the populations' fitness and genetic diversity. However, it should be noted that there were no specimens sampled from the supposed geographical barrier, hence it is possible that the reported gap may be due to unsampled populations from that area. More samples may prove the presence of seasonal split between *C. nanum* populations as observed in *Strongylopus grayii* (Tolley *et al.*, 2010). Thus, future studies should incorporate samples from the whole distribution range of *C. nanum* within South Africa.

#### 4.4.2 Population structure

Among *C. nanum* populations, nucleotide diversity was low whereas genetic diversity was generally high except for two populations, signalling a sudden population expansion (Avise, 2000). Hogsback and Katberg (artificial pan) populations had no genetic diversity possibly reflecting inbreeding and loss of effective population size and population persistence (Ellstrand and Elam, 1993; Saccheri *et al.*, 1998; Byers and Waller, 1999; Frankham *et al.*, 2002). This pattern is concordant with large populations as they are less sensitive to random loss of genetic variation by genetic drift and thus retain ancestral signatures (Ellegren, 2009). The above is also corroborated by the presence of low genetic variation between populations and high degree of shared haplotypes within the sampled populations. This, coupled with variation within sample populations accounting for more of the total genetic variation than among populations, suggests substantial sub-structuring within *C. nanum* populations, indicating that gene flow is indeed high, as anticipated from a habitat generalist. This is



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plausible given the reproductive cycle of *C. nanum* is solely dependent on water, hence periodic and seasonal rains and flooding events can play a key role in connecting populations, accommodating migrants and maintaining effective population sizes. Most of the populations within *C. nanum* exhibited no evidence of selection due to low sample size as presented by Tajima's  $D$  and Fu's  $F_s$  indices equal to zero. However, it should be noted that 70% of the populations had a sample size of five or less. Thus, low sample size (Fu and Li, 1993) is more likely causative for the demographic history recovered for the *C. nanum* populations.

The uncorrected sequence divergence between clade 1 (southern distribution) and clade 2 (northern distribution) was 3.10%. These divergences are comparable to those found between clades of other frogs (e.g. Roberts *et al.*, 2006; Liu *et al.*, 2015) and other similar taxon, (e.g. *Salamander atra*: Riberon *et al.*, 2001), indicating that these two clades are conspecifics within *C. nanum* (see Kartavtsev, 2011). Given that *Cyt b* locus revolves twice as much as 16S rRNA gene, the 3.10% *Cyt b* sequence divergence between the two clades corresponds to a 16S rRNA divergence of 1.55%. Based on these data, it seems reasonable to consider these two clades as conspecifics as the values are low, compared with those used to delimit other anuran taxa (e.g. Fouquet *et al.*, 2007b). Interestingly, lower divergences especially within the genus, *Cacosternum* have been used, together with other additional characters provided (Channing *et al.*, 2013b). However, sequence divergence alone cannot be used to determine species boundaries (Ferguson, 2002; de Queiroz, 2007a) and other data including bioacoustics, morphology, landscape genetics should be incorporated to validate the species taxonomic standing (Vieites *et al.*, 2009).

#### **4.5 The resolution power of mtDNA compared to nuDNA in population genetics**

The nuclear locus, Rhodopsin revealed deeper relationships within the study species compared to RAG1, which also had less amplification and sequencing success. Overall, phylogenetic and phylogeographic analyses using nuDNA loci for *Anhydrophryne rattrayi* indicated a similarity in paternal ancestry between populations from Katberg, Hogsback, Kologha and Kubusi with the Isidenge population revealing a separate and distinct paternal heritage. For *Arthroleptis wahlbergii*, TCS analyses using Rhod retrieved a single but connected haplotype network comprising specimens from the four Eastern Cape forests suggesting a paternal connection between all localities. *Cacosternum nanum*, however, showed the most interesting paternal genetic structure. From the 25 specimens sequenced, selecting one per locality, four (Mbotyi camp, Mbotyi quarry, Main beach road, Mkambati

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N.R and Baziya) were not connected to the rest. Instead, they occurred as single haplotypes except for Mbotyi quarry and Mbotyi camp which shared a haplotype. This indicates that these four populations may come from different patrilineal lineages, separate from the rest, insighting possible hybridisation or incomplete lineage sorting. Two out of the five specimens from the southern distribution (clade 1) failed to amplify but the three that did, related to the rest of the populations, indicating an absence of a barrier. Discordance between mtDNA and nuDNA is expected given the lower effective size of mtDNA, thus stronger genetic drift (Canestrelli *et al.*, 2007). Therefore, these results suggest that future studies should consider the inclusion of several nuclear and mtDNA loci for complete phylogeographic representation and conclusive inferences to be made (Edwards and Beerli, 2000; Zhang and Hewitt, 2003; Channing *et al.*, 2013b; Conradie, 2014; Loader *et al.*, 2015; Hung *et al.*, 2016)).

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# **CHAPTER FIVE CONCLUSIONS AND RECOMMENDATIONS**

## Chapter 5

### Conclusions and Recommendations

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#### 5. Conclusions

##### 5.1 Conservation implications

The aim of the thesis was to shed light on the evolutionary relationships among three anuran taxa known to exhibit different habitat preferences within the Eastern Cape Province forests. The study clearly demonstrated high genetic population structure and exhibited older cladogenic activity in the two forest dwelling specialist species *A. rattrayi* and *A. wahlbergii*. In contrast, the generalist *C. nanum* indicated shallow genetic differentiation together with recent Late Quaternary divergences. While the mtDNA loci revealed deep structure, the nuDNA loci revealed limited genetic differentiation. These results suggest that refugial habitats, assuming they are not threatened by further fragmentation through exposure to climatic or anthropogenic changes, ought to be major conservation priorities, especially so for the IUCN listed (vulnerable) species; the taxon may be able to persist and maintain stable populations through time. Thus, I propose that the clades in each of the forest dwelling taxa, at least be regarded as management units (MU) since they are only distinct based on the mtDNA data and do not constitute Evolutionary Significant Units (ESU's) (Crandall *et al.*, 2000). These MU's will especially be useful and applicable to Kologha and Kubusi forests as *A. rattrayi* populations from those localities seem to have some degree of gene flow which tends to increase genetic diversity and essentially, fitness within the species. Population-based conservation strategies are essential given the fragmentation and isolation of forest extent in South Africa. For small and moisture dependent species like anurans that have a low recruitment of dispersing individuals (Bulger *et al.*, 2003; Cushman, 2006), effective conservation planning will require improvements in information dissemination between scientists and forest managers on factors that influence species survival and persistence in these fragmented habitats. This will require extensive use of feedback loops between the several management units and the need for comprehensive research on relationships between land use patterns and anuran population sizes, reproduction mechanisms and dispersal across different scales to elucidate functional conservation goals.

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## 5.2 Knowledge gaps and recommendations

There are several vital limitations to this study. Sample size was small for all three-anuran species due to several reasons. Firstly, *A. rattrayi* is a terrestrial leaf litter frog which favours moss vegetation (reproduction, safety and moisture) and due to their small size, even if they call, triangulating the call is difficult, and in the process, their habitat is often destroyed. Regardless, the study was able to rediscover a population from Kologha that was last seen in the 1960's (Werner Conradie, personal communication) and a new record from Kubusi. Second, *A. wahlbergii* individuals are usually identifiable by call, hence finding samples when it is not raining is often unsuccessful hence most samples were collected during rainfall events. Lastly, the complete distribution range of *C. nanum* within the Eastern Cape Province was not covered due to logistical purposes. The study made use of both mtDNA loci and nuDNA loci although only one sequence per locality was analysed for the latter and data was treated separately and not included as in a total evidence dataset. The discordance between mtDNA and nuDNA loci, although expected could be counteracted by making use of other sensitive gene markers and integrating a multi-locus approach could help in resolving phylogenetic relationships (Mallo and Posada, 2016), especially for generalist species like *C. nanum* which revealed unresolved genetic structure among clades. The study lacked the co-distribution aspect necessary for a complete comparative phylogeographic study to be done. This was due to the lack of samples of the generalist species, *C. nanum* from localities where the two forest endemics occur. Thus, increases in sample size, coupled with targeted sampling regimes are suggested for future work. While the study provided useful insights into the discovery of putative species using mtDNA, species delimitation using genetic data alone is not encouraged (Sites and Crandall, 1997; Vieites *et al.*, 2009; Feinberg *et al.*, 2014; Priti *et al.*, 2016). Thus, several lines of evidence including, but not restricted to the following: advertisement calls (bioacoustics) should be recorded wherever necessary, especially during mating season as males tend to call often and voucher specimens should be collected from all representative populations and analysed for morphological differences as well as the incorporation of habitat/ landscape genetics.

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**Appendix 1: List of voucher specimens collected during the study stored in herpetological collections of the Port Elizabeth Museum, Bayworld (PEM), South Africa.**

N	Field No.	Species	PEM No.	Latitude	Longitude	Locality
1	AR01	<i>Anhydrophryne rattrayi</i>	PEM A11747	-32.595350	26.955700	Hogsback1
2	AR02	<i>Anhydrophryne rattrayi</i>	PEM A11748	-32.595350	26.955700	Hogsback2
3	WC-4177	<i>Anhydrophryne rattrayi</i>	PEM A11772	-32.687970	27.278560	Isidenge1
4	WC-4180	<i>Anhydrophryne rattrayi</i>	PEM A11774	-32.687970	27.278560	Isidenge3
5	WC-4182	<i>Anhydrophryne rattrayi</i>	PEM A11773	-32.687970	27.278560	Isidenge4
6	WC-4183	<i>Anhydrophryne rattrayi</i>	PEM A11775	-32.687970	27.278560	Isidenge5
7	WC-4185	<i>Anhydrophryne rattrayi</i>	PEM A11776	-32.687970	27.278560	Isidenge2
8	WC-4188	<i>Anhydrophryne rattrayi</i>	PEM A11777	-32.473550	26.672510	Katberg1
9	WC-5443	<i>Anhydrophryne rattrayi</i>	PEM A12290	-32.536171	27.363178	Kologha8
10	WC-5444	<i>Anhydrophryne rattrayi</i>	PEM A12291	-32.536171	27.363178	Kologha9
11	WC-5452	<i>Anhydrophryne rattrayi</i>	PEM A TBA	-32.532517	27.363641	Kologha10
12	WC-5453	<i>Anhydrophryne rattrayi</i>	PEM A TBA	-32.532517	27.363641	Kologha11
13	WC-5454	<i>Anhydrophryne rattrayi</i>	PEM A TBA	-32.532517	27.363641	Kologha12
14	WC-5455	<i>Anhydrophryne rattrayi</i>	PEM A TBA	-32.532517	27.363641	Kologha13
15	WC-5456	<i>Anhydrophryne rattrayi</i>	PEM A TBA	-32.687855	27.277444	Isidenge11
16	WC-5457	<i>Anhydrophryne rattrayi</i>	PEM A TBA	-32.558684	27.315337	Kubusi2
17	WC-5458	<i>Anhydrophryne rattrayi</i>	PEM A TBA	-32.558684	27.315337	Kubusi3
18	WC-4103	<i>Arthroleptis wahlbergii</i>	PEM A11846	-31.428025	29.726147	Mbotyi5
19	WC-4104	<i>Arthroleptis wahlbergii</i>	PEM A11847	-31.428025	29.726147	Mbotyi6
20	WC-4105	<i>Arthroleptis wahlbergii</i>	PEM A11848	-31.428025	29.726147	Mbotyi7
21	WC-4357	<i>Arthroleptis wahlbergii</i>	PEM A11849	-31.428025	29.726147	Mbotyi1
22	WC-4358	<i>Arthroleptis wahlbergii</i>	PEM A11850	-31.428025	29.726147	Mbotyi2
23	WC-4359	<i>Arthroleptis wahlbergii</i>	PEM A11851	-31.428025	29.726147	Mbotyi3
24	WC-4360	<i>Arthroleptis wahlbergii</i>	PEM A11852	-31.428025	29.726147	Mbotyi4
25	WC-4368	<i>Arthroleptis wahlbergii</i>	PEM A11849	-31.428025	29.726147	Mbotyi8
26	WC-4371	<i>Arthroleptis wahlbergii</i>	PEM A11855	-31.428025	29.726147	Mbotyi 9
27	WC-4372	<i>Arthroleptis wahlbergii</i>	PEM A11856	-31.428025	29.726147	Mbotyi 10
28	WC-4379	<i>Arthroleptis wahlbergii</i>	PEM A11857	-31.433714	29.633682	Goso1
29	WC-4380	<i>Arthroleptis wahlbergii</i>	PEM A11858	-31.433714	29.633682	Goso2
30	WC-4381	<i>Arthroleptis wahlbergii</i>	PEM A11859	-31.433714	29.633682	Goso3



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**Appendix 1: continues**

<b>N</b>	<b>Field No.</b>	<b>Species</b>	<b>PEM No.</b>	<b>Latitude</b>	<b>Longitude</b>	<b>Locality</b>
31	WC-4382	<i>Arthroleptis wahlbergii</i>	PEM A11860	-31.433714	29.633682	Goso4
32	WC-4384	<i>Arthroleptis wahlbergii</i>	PEM A11862	-31.433714	29.633682	Goso5
33	WC-4385	<i>Arthroleptis wahlbergii</i>	PEM A11863	-31.433714	29.633682	Goso6
34	WC-4386	<i>Arthroleptis wahlbergii</i>	PEM A11864	-31.433714	29.633682	Goso7
35	WC-4387	<i>Arthroleptis wahlbergii</i>	PEM A11865	-31.433714	29.633682	Goso8
36	WC-4388	<i>Arthroleptis wahlbergii</i>	PEM A11866	-31.433714	29.633682	Goso9
37	WC-4389	<i>Arthroleptis wahlbergii</i>	PEM A11867	-31.433714	29.633682	Goso10
38	WC-4390	<i>Arthroleptis wahlbergii</i>	PEM A11868	-31.433714	29.633682	Goso11
39	WC-4419	<i>Arthroleptis wahlbergii</i>	PEM A11869	-31.433714	29.633682	Goso13
40	WC-4420	<i>Arthroleptis wahlbergii</i>	PEM A11870	-31.433714	29.633682	Goso12
41	WC-DNA-389	<i>Arthroleptis wahlbergii</i>	PEM A9967	-31.818080	29.300170	Hluleka N.R1
42	WC-DNA-714	<i>Arthroleptis wahlbergii</i>	PEM A10550	-31.651030	29.511920	Silaka N.R1
43	WC-DNA-746	<i>Arthroleptis wahlbergii</i>	PEM A10553	-31.651030	29.511920	Silaka N.R2
44	WC-DNA-747	<i>Arthroleptis wahlbergii</i>	PEM A10552	-31.651030	29.511920	Silaka N.R3
45	WC-DNA-820	<i>Arthroleptis wahlbergii</i>	PEM A10548	-31.649540	29.505190	Silaka N.R7
46	WC-DNA-821	<i>Arthroleptis wahlbergii</i>	PEM A10554	-31.649540	29.505190	Silaka N.R6
47	WC-DNA-825	<i>Arthroleptis wahlbergii</i>	PEM A10549	-31.655310	29.504570	Silaka N.R5
48	WC-DNA-853	<i>Arthroleptis wahlbergii</i>	PEM A10551	-31.651030	29.511920	Silaka N.R4
49	WC-3621	<i>Cacosternum nanum</i>	PEM A11622	-31.229450	27.515436	Otto du Plessis
50	PEM_D0016	<i>Cacosternum nanum</i>	N/A	-33.976471	23.561055	Natures valley1
51	PEM_D0018	<i>Cacosternum nanum</i>	N/A	-33.976471	23.561055	Natures valley2
52	WC-2698	<i>Cacosternum nanum</i>	PEM A11465	-34.008268	25.666595	NMU South campus
53	WC-2738	<i>Cacosternum nanum</i>	PEM A11416	-32.669020	26.479930	Katberg artificial pan1
54	WC-2739	<i>Cacosternum nanum</i>	PEM A11417	-32.669020	26.479930	Katberg artificial pan2
55	WC-2800	<i>Cacosternum nanum</i>	PEM A11449	-32.669020	26.479930	Katberg road
56	WC-3799	<i>Cacosternum nanum</i>	PEM A11786	-32.678000	26.511902	Fort Fordyce1
57	WC-3818	<i>Cacosternum nanum</i>	PEM A11787	-32.403070	27.448510	Kubusi3
58	WC-3819	<i>Cacosternum nanum</i>	PEM A11788	-32.685638	26.497973	Fort Fordyce2
59	WC-4097	<i>Cacosternum nanum</i>	PEM A11883	-31.296539	29.975944	Forest Swamp1, Mkambati N.R
60	WC-4098	<i>Cacosternum nanum</i>	PEM A11884	-31.296539	29.975944	Forest Swamp2, Mkambati N.R
61	WC-4099	<i>Cacosternum nanum</i>	PEM A11885	-31.296539	29.975944	Forest Swamp3, Mkambati N.R
62	WC-4100	<i>Cacosternum nanum</i>	PEM A11886	-31.296539	29.975944	Forest Swamp4, Mkambati N.R
63	WC-4101	<i>Cacosternum nanum</i>	PEM A11887	-31.296539	29.975944	Forest Swamp5, Mkambati N.R

Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.

**Appendix 1: continues**

<b>N</b>	<b>Field No.</b>	<b>Species</b>	<b>PEM No.</b>	<b>Latitude</b>	<b>Longitude</b>	<b>Locality</b>
64	WC-4102	<i>Cacosternum nanum</i>	PEM A11888	-31.296539	29.975944	Forest Swamp6, Mkambati N.R
65	WC-4112	<i>Cacosternum nanum</i>	PEM A11876	-31.451467	29.732108	Mbotyi quarry1
66	WC-4159	<i>Cacosternum nanum</i>	PEM A11789	-32.403066	27.448510	Kubusi1
67	WC-4181	<i>Cacosternum nanum</i>	PEM A11790	-32.403066	27.448510	Kubusi2
68	WC-4356	<i>Cacosternum nanum</i>	PEM A11877	-31.407421	29.407421	Goso1
69	WC-4399	<i>Cacosternum nanum</i>	PEM A11878	-31.433714	29.633682	Goso3
70	WC-4400	<i>Cacosternum nanum</i>	PEM A11879	-31.433714	29.633682	Goso2
71	WC-4418	<i>Cacosternum nanum</i>	PEM A11881	-31.465205	29.730399	Mbotyi campsite1
72	WC-4453	<i>Cacosternum nanum</i>	PEM A11882	-31.294009	29.929676	Superbowl area, Mkambati N.R
73	WC-4455	<i>Cacosternum nanum</i>	PEM A11889	-31.296539	29.975944	Forest Swamp7, Mkambati N.R
74	WC-4460	<i>Cacosternum nanum</i>	PEM A11890	-31.296539	29.975944	Forest Swamp8, Mkambati N.R
75	WC-4463	<i>Cacosternum nanum</i>	PEM A11891	-31.296539	29.975944	Forest Swamp9, Mkambati N.R
76	WC-4471	<i>Cacosternum nanum</i>	PEM A11896	-31.307355	29.976235	Main beach road1, Mkambati N.R
77	WC-4472	<i>Cacosternum nanum</i>	PEM A11897	-31.307355	29.976235	Main beach road2, Mkambati N.R
78	WC-4473	<i>Cacosternum nanum</i>	PEM A11898	-31.307355	29.976235	Main beach road3, Mkambati N.R
79	WC-4478	<i>Cacosternum nanum</i>	PEM A11892	-31.296539	29.975944	Forest Swamp10, Mkambati N.R
80	WC-4497	<i>Cacosternum nanum</i>	PEM A11893	-31.296539	29.975944	Forest Swamp11, Mkambati N.R
81	WC-4498	<i>Cacosternum nanum</i>	PEM A11894	-31.296539	29.975944	Forest Swamp12, Mkambati N.R
82	WC-4915	<i>Cacosternum nanum</i>	PEM A12052	-31.580656	28.391107	Baziya1
83	WC-4916	<i>Cacosternum nanum</i>	PEM A12047	-31.567650	28.429241	Baziya2
84	WC-4917	<i>Cacosternum nanum</i>	PEM A12048	-31.567650	28.429241	Baziya3
85	WC-4918	<i>Cacosternum nanum</i>	PEM A12049	-31.567650	28.429241	Baziya4
86	WC-4919	<i>Cacosternum nanum</i>	PEM A12050	-31.567650	28.429241	Baziya5
87	WC-4920	<i>Cacosternum nanum</i>	PEM A12051	-31.567650	28.429241	Baziya6
88	WC-4929	<i>Cacosternum nanum</i>	PEM A12059	-31.412500	28.732900	Nqadu1
89	WC-4931	<i>Cacosternum nanum</i>	PEM A12060	-31.412500	28.732900	Nqadu2
90	WC-4933	<i>Cacosternum nanum</i>	PEM A12061	-31.412500	28.732900	Nqadu3
91	WC-4934	<i>Cacosternum nanum</i>	PEM A12062	-31.412500	28.732900	Nqadu4
92	WC-4936	<i>Cacosternum nanum</i>	PEM A12063	-31.412500	28.732900	Nqadu5
93	WC-4938	<i>Cacosternum nanum</i>	PEM A12064	-31.412500	28.732900	Nqadu6
94	WC-4940	<i>Cacosternum nanum</i>	PEM A12055	-31.412500	28.732900	Nqadu16
95	WC-4941	<i>Cacosternum nanum</i>	PEM A12065	-31.412500	28.732900	Nqadu7
96	WC-4942	<i>Cacosternum nanum</i>	PEM A12066	-31.412500	28.732900	Nqadu8

Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.

**Appendix 1: continues**

<b>N</b>	<b>Field No.</b>	<b>Species</b>	<b>PEM No.</b>	<b>Latitude</b>	<b>Longitude</b>	<b>Locality</b>
97	WC-4943	<i>Cacosternum nanum</i>	PEM A12067	-31.412500	28.732900	Nqadu9
98	WC-4944	<i>Cacosternum nanum</i>	PEM A12068	-31.412500	28.732900	Nqadu10
99	WC-4945	<i>Cacosternum nanum</i>	PEM A12069	-31.412500	28.732900	Nqadu11
100	WC-4946	<i>Cacosternum nanum</i>	PEM A12070	-31.412500	28.732900	Nqadu12
101	WC-4947	<i>Cacosternum nanum</i>	PEM A12071	-31.412500	28.732900	Nqadu13
102	WC-4948	<i>Cacosternum nanum</i>	PEM A12072	-31.412500	28.732900	Nqadu14
103	WC-4949	<i>Cacosternum nanum</i>	PEM A12073	-31.412500	28.732900	Nqadu15
104	WC-4953	<i>Cacosternum nanum</i>	PEM A12053	-31.415000	28.734500	Nqadu17
105	WC-4954	<i>Cacosternum nanum</i>	PEM A12054	-31.415000	28.734500	Nqadu18
106	WC-4977	<i>Cacosternum nanum</i>	PEM A12056	-31.162206	28.585390	Ntywenka Plantation1
107	WC-4978	<i>Cacosternum nanum</i>	PEM A12057	-31.162206	28.585390	Ntywenka Plantation2
108	WC-4979	<i>Cacosternum nanum</i>	PEM A12058	-31.162206	28.585390	Ntywenka Plantation3
109	WC-5000	<i>Cacosternum nanum</i>	PEM A11958	-32.598007	26.571902	Mpofu N.R1
110	WC-5017	<i>Cacosternum nanum</i>	PEM A11959	-32.598028	26.571841	Mpofu N.R2
111	WC-5279	<i>Cacosternum nanum</i>	PEM A12299	-32.303578	28.828701	Dwesa N.R9
112	WC-5280	<i>Cacosternum nanum</i>	PEM A12300	-32.303578	28.828701	Dwesa N.R10
113	WC-5285	<i>Cacosternum nanum</i>	PEM A12301	-32.271116	28.843800	Dwesa N.R11
114	WC-5298	<i>Cacosternum nanum</i>	PEM A12302	-32.271116	28.843800	Dwesa N.R12
115	WC-5315	<i>Cacosternum nanum</i>	PEM A12303	-32.275967	28.848640	Dwesa N.R8
116	WC-5357	<i>Cacosternum nanum</i>	PEM A12304	-32.440738	28.604258	Manubi1
117	WC-5360	<i>Cacosternum nanum</i>	PEM A12305	-32.453375	28.607378	Manubi2
118	WC-5366	<i>Cacosternum nanum</i>	PEM A12306	-32.440513	28.617701	Manubi3
119	WC-5367	<i>Cacosternum nanum</i>	PEM A12307	-32.440513	28.617701	Manubi4
120	WC-5368	<i>Cacosternum nanum</i>	PEM A12308	-32.440513	28.617701	Manubi5
121	WC-5369	<i>Cacosternum nanum</i>	PEM A12309	-32.440513	28.617701	Manubi6
122	WC-5659	<i>Cacosternum nanum</i>	PEM A12401	-33.979184	25.372133	The Island N. R1
123	WC-5660	<i>Cacosternum nanum</i>	PEM A12402	-33.979184	25.372133	The Island N. R2
124	WC-5662	<i>Cacosternum nanum</i>	PEM A12404	-33.979184	25.372133	The Island N.R3
125	WC-5663	<i>Cacosternum nanum</i>	PEM A12405	-33.979184	25.372133	The Island N.R4
126	WC-5668	<i>Cacosternum nanum</i>	PEM A12406	-33.985275	25.377925	The Island N.R5
127	WC-DNA-101	<i>Cacosternum nanum</i>	PEM A9659	-34.002222	25.527250	Lovemore heights
128	WC-DNA-306	<i>Cacosternum nanum</i>	PEM A10076	-32.560305	26.913851	Amatole forest1
129	WC-DNA-315	<i>Cacosternum nanum</i>	PEM A10075	-32.560305	26.913851	Amatole forest2

Comparative phylogeography of three anuran species in the Eastern Cape Province forests, South Africa.

**Appendix 1: continues**

<b>N</b>	<b>Field No.</b>	<b>Species</b>	<b>PEM No.</b>	<b>Latitude</b>	<b>Longitude</b>	<b>Locality</b>
130	WC-DNA-425	<i>Cacosternum nanum</i>	PEM A9999	-31.828220	29.296820	Hluleka N.R1
131	WC-DNA-456	<i>Cacosternum nanum</i>	PEM A10149	-32.287920	28.867940	Dwesa N.R4
132	WC-DNA-644	<i>Cacosternum nanum</i>	PEM A10152	-32.287920	28.867940	Dwesa N.R3
133	WC-DNA-653	<i>Cacosternum nanum</i>	PEM A10143	-32.302780	28.870350	Kobolo2
134	WC-DNA-656	<i>Cacosternum nanum</i>	PEM A10154	-32.287920	28.867940	Dwesa N.R5
135	WC-DNA-671	<i>Cacosternum nanum</i>	PEM A10146	-32.302780	28.870350	Kobolo1
136	WC-DNA-672	<i>Cacosternum nanum</i>	PEM A10150	-32.283040	28.864690	Dwesa N.R6
137	WC-DNA-707	<i>Cacosternum nanum</i>	PEM A10643	-32.579722	26.943056	Hogsback2
138	WC-DNA-739	<i>Cacosternum nanum</i>	PEM A10574	-31.654080	29.506020	Dwesa N.R2
139	WC-DNA-749	<i>Cacosternum nanum</i>	PEM A10571	-31.653860	29.506830	Dwesa N.R1
140	WC-DNA-770	<i>Cacosternum nanum</i>	PEM A10683	-32.037500	29.108333	Hole in wall
141	WC-DNA-780	<i>Cacosternum nanum</i>	PEM A10572	-31.653300	29.506820	Silaka N.R3
142	WC-DNA-788	<i>Cacosternum nanum</i>	PEM A10575	-31.655310	29.504570	Silaka N.R1
143	WC-DNA-797	<i>Cacosternum nanum</i>	PEM A10573	-31.652380	29.507070	Silaka N.R2
144	WC-DNA-814	<i>Cacosternum nanum</i>	PEM A10642	-32.579722	26.943056	Hogsback1
145	WC-DNA-864	<i>Cacosternum nanum</i>	PEM A10576	-31.655310	29.504570	Silaka N.R4
146	WC-DNA-904	<i>Cacosternum nanum</i>	PEM A10637	-33.654028	25.246111	Eels cave