Phylogeography and speciation in the genus *Arthroleptella*

Andrew Alexander Turner

A thesis submitted in fulfilment of the requirements for the degree of
Doctor Philosophiae in the Department of Biodiversity and Conservation Biology,
University of the Western Cape.

November 2010

Supervisor: Prof A. Channing

UNIVERSITY of the
WESTERN CAPE

Arthroleptella villiersi from Paarl Mountain

KEYWORDS

Moss frogs, phylogeography, phylogeny, mitochondrial genes, nuclear genes, speciation, Cape Floral Region, Cape Fold Mountains, advertisement calls, distribution, threat status, fire, alien invasive species, morphology, habitat requirements.

ABSTRACT

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A.A. Turner

PhD Thesis, Department of Biodiversity and Conservation Biology, University of the Western Cape

Moss frogs are restricted to permanently moist terrestrial habitats in the south-western Cape Fold Mountains. There is a very close association between *Arthroleptella* distribution and Table Mountain Sandstone. Suitable habitats are generally occupied by allopatric populations of moss frogs. Comprehensive spatial sampling of moss frogs (genus *Arthroleptella*) in the Cape Floristic Region biodiversity hotspot yielded 192 new distribution records; 5 842 advertisement calls from 240 individual male frogs; 31 Rag-1, 76 16S, 54 12S sequences and morphological measurements of 90 specimens. There are many differences in male advertisement call and genetic sequences between populations on different mountain ranges, even over small distances. A mitochondrial and nuclear gene phylogeny of the southern African Pyxicephalidae places *Natalobatrachus* as the sister genus to *Arthroleptella*. Application of a Bayesian relaxed molecular clock model indicates that *Arthroleptella* arose between 20 and 39 Ma. Phylogenetic trees return two main clades within *Arthroleptella*: one consists of species which exhibit chirp-like calls and the second contains species with longer calls composed of a series of clicks. These two clades diverged between 19 and 22 Ma. There is a general pattern of strong phylogeographic structure with many small, isolated populations. Three species are identified within the Chirping clade and seven in the Clicking clade, including three undescribed species. This population structure is a result of the patchy distribution of suitable habitat and low vagility of the moss frogs. The distribution and speciation of moss frogs has been affected by drying and cooling climate change, changing geomorphology over the last 20 Ma and the increasing prevalence of fire over the last 5 Ma. An assessment of the threat status of each species according to IUCN criteria categorised one species as Least Concern, seven as Near Threatened, one as Vulnerable and one as Critically Endangered. The primary threats to *Arthroleptella* are invasive alien plants and increased fire frequencies and intensities.

DECLARATION

I declare that this thesis is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Andrew Alexander Turner Date

Signed

ACKNOWLEDGEMENTS

I wish to thank the following people for their assistance in the completion of the thesis: Alan Channing for supervising and supporting this work. Sarah Davies for her enthusiasm, encouragement and support from field work to formatting. For discussions and assistance on the technicalities of sequencing genes and their meanings I am grateful to Krystal Tolley, Michael Cunningham, Daniel Goedbloed, Abeda Dawood, Gill Baker and John Measey.

For access to sampling localities I thank Michael Swart of Swartberg, Carel le Roux of Rhodebloem, Michael Stuttaford of Driekoppe, Victor Sperling of Delheim, Raymond O'Grady of Hillcrest, Giorgio Lombardi of Vogelgat, Christo du Plessis of Jonaskop, Mr J. Roux of Kaimansgat and Chad Cheney of Table Mountain National Park.

For assistance in the field I thank my CapeNature colleagues and in particular Atherton de Villiers, Riki de Villiers, Micheal Lewis, Peter Viljoen, Afrika de Bruyn, Marius Brand, Deon Rossouw, Mark Johns, Leon Lourens, Ivan Jansen, Nicolaas Swarts, Adam Jacobs and John Davids. Prof. Eddie van Dijk provided x-ray facilities and gracious assistance in operating the equipment and obtaining the images. I also thank Marius Burger for sharing his experience and for assistance in the field. Finally, I thank Ernst Baard for providing the opportunity to complete the thesis within the demands of full time employment.

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LIST OF ABBREVIATIONS

12S 12S ribosomal subunit

16S 16S ribosomal subunit

CFR Cape Floral Region

CFM Cape Fold Mountains

DIVA Dispersal-vicariance analysis

EL Eyelid width

EY Eye width

FM Femur length

FO Foot length

HB Head-body length = snout–urostyle length)

HW Head width

IN Internarial distance

IO Interorbital distance

ka thousand years ago

SN Snout length

Mt Mitochondrial

Ma Million years ago

MP Maximum parsimony

AMOVA Analysis of molecular variation

ND2 Sodium Dehydrogenase subunit 2

Nu Nuclear

PCR Polymerase chain reaction

Rag-1 Recombinase activating gene 1

Rag-2 Recombinase activating gene 1

SAMOVA Spatial analysis of molecular variation

TB Tibia length

TMG Table Mountain Group

TMS Table Mountain Sandstone

TO Toe length

Tyr Tyrosinase precursor

Rhod Rhodopsin

WCP Western Cape Province

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Chapter 1. Introduction

1.1 Motivation

Moss frogs of the genus *Arthroleptella* are habitat specialists restricted to permanently moist areas in the Cape Floral Region (CFR) which are very patchily distributed. Taxonomic work prior to this study has revealed the existence of several cryptic species even though moss frogs had not been sampled or recorded from many mountain ranges where they are expected to occur. These facts indicated that their distribution and systematics were incompletely known and that a revision of the distribution and taxonomy of the genus was required. This study addresses these information gaps by providing a comprehensive spatial sampling across the range of this genus. Documentation of biodiversity and its distribution is a primary step for assessing extinction threats and developing management plans to direct conservation action. Underestimation of anuran biodiversity in tropical regions such as Madagascar may be as much as 100 % (Vieites *et al.* 2009). Such a high level of underestimation is not expected with anurans in the CFR but there appears to be substantial room for improving our knowledge of the full suite of anuran diversity in this region. This study contributes to the request for empirical phylogeographic surveys in regions of the Southern Hemisphere (Beheregaray 2008).

1.2 Moss frogs

Moss frogs are part of the family Pyxicephalidae (Frost *et al.* 2006) a unique African family of frogs that has diversified into a large number of genera and species (see Van der Meijden *et al.* 2005). Moss frogs are very small, adult snout-urostyle length 12-22 mm (Hewitt 1926; 1935; Channing *et al*. 1994; Dawood & Channing 2000; Turner *et al*. 2004; Turner & Channing 2008), secretive frogs that live in wet, mossy places associated with mountains (Channing 2001).

Within the WCP, the genus is restricted to the south western parts of the Cape Fold Mountains (CFM) and their distribution is represented by a roughly ‘L’ shaped pattern from the Groot Winterhoek Mountains near Porterville in the north (Turner *et al.* 2004) to Table Mountain and other the slopes of the Cape Peninsula in the south and east to the Riviersonderend Mountains (Channing *et al.* 1994).

There has been considerable taxonomic confusion in the past e.g. (Poynton 1964; Passmore & Carruthers 1995) which has obscured the biographical patterns within *Arthroleptella* (see section on Systematic History below). Although this situation was tackled by Channing *et al.* (1994) and Dawood & Channing (2000), gaps still remained in our knowledge of alpha taxonomy, distributions, habitat associations and calling behaviour of moss frogs. The patchy nature of their

distribution at a range of geographical scales from mountain ranges to individual seeps, along with the difficulties associated with identifying moss frog species called out for further attention to be given to this genus. Many of the suitable habitats are situated on the slopes or summits of mountains which are difficult to access. This has made complete sampling across the full distribution of the genus difficult and time-consuming. This study is an attempt at providing a geographically complete sampling of the genus.

Moss frogs have been said to be 'morphologically indistinguishable' by Channing *et al.* (1994). Morphological similarity has disguised many cryptic frog species for those workers relying on the use of morphological characters to separate species. This study will revisit the morphology of *Arthroleptella* to test the Channing *et al.* (1994) hypothesis of morphological similarity and to establish if indeed certain morphological characters do have systematic value and if so, to what extent.

Many South African amphibians still require alpha taxonomic work (Channing 2005). The study of moss frogs have benefited from the relatively recent systematic investigations of Channing *et al.* (1994), Dawood & Channing (2000), Turner *et al*. (2004), Dawood & Stam (2006) and Turner & Channing (2008). Despite this work, the complexity of the systematics of this genus still requires full description. It is my intention here to provide a thorough review of the systematics of *Arthroleptella* and to provide an evolutionary context for the pattern of speciation in this genus.

The advertisement calls of male frogs have been used very successfully as species specific characters (e.g. Noble & Noble 1923 cited in Rand 2001; Heyer *et al.* 1996; Rand 2001; Littlejohn 1965; Passmore 1977; Telford & Passmore 1981) although see Wollenberg *et al.* (2007). It should also be noted from an evolutionary process perspective that species recognition and mate choice among conspecific and heterospecifics cannot be separated (Backwell & Jennions 1993). The utility of this character derives from the fact that calls are used to attract mates (Wells 1977) and so are a direct precursor to mating and gene flow. The advertisement calls of moss frogs have been used to distinguish species (Channing *et al.* 1994, Dawood & Channing 2000, Turner *et al.* 2004, Turner & Channing 2008) and advertisement calls are examined across the genus *Arthroleptella* in this study. The utility of advertisement calls in defining species concepts is discussed further below.

The use of morphological characters in taxonomy and systematics dates back to the origin of these endeavours and continues to be useful, if carefully applied and/or combined with molecular data (e.g. De Sá & Hillis 1990; Emerson *et al.* 2000). The use of molecular analysis of fragments of the

genome to assess phylogenetic relationships is now routine practice (the use of entire genomes is still in an early stage of development e.g. (Li *et al.* 2002; Sims *et al.* 2009)). Mitochondrial DNA is widely used as it has the useful property of not being subject to recombination as it is inherited only through the maternal line. However, mitochondrial DNA is not selectively neutral (Dowling *et al.* 2008). A disadvantage of this feature is that only matrilineal ancestry is represented (although among some non-vertebrate taxa there are non-maternal modes of mitochondrial inheritance but see Bromham *et al.* (2003)). Recent anuran phylogenies based on DNA sequences use mitochondrial and nuclear genes fragments e.g. (Cunningham & Cherry 2004; Faivovich *et al.* 2005; Bossuyt *et al.* 2006; Frost *et al.* 2006; Glaw & Vences 2006; Che *et al.* 2007).

The current study uses a combination of advertisement call, DNA sequence data and morphological data to build a comprehensive phylogeny of the genus *Arthroleptella*.

1.3 Biogeographic context

The Cape Floral Region is a well-known biodiversity hotspot (Myers 1990; Myers *et al.* 2000). The CFR is incorporated in the more extensive fynbos biome or Cape Floral Kingdom (CFK) but is functionally synonymous with the CFK as very few additional vegetation types occur in the more extensive definition of the latter (see Figure 1 for location). The CFR is recognised as a biodiversity hotspot because of the enormous density of plant species (over 8 900 in only 78 555 km^2) making it the world's smallest floral kingdom (Cowling & Richardson 1995). The CFR's fame for its floral wealth has recently been augmented by a growing body of work revealing that the fauna is more diverse than previously realized e.g. dwarf chameleons (Tolley *et al.* 2004; Branch *et al.* 2006); cordylid lizards (Daniels *et al.* 2004); dragonflies (Dijkstra *et al.* 2007); cicadas (Price *et al.* 2007); elephant-shrews (Smit *et al.* 2008) and dwarf mountain toads (Tolley *et al.* 2010b).

The fynbos biome experiences a typical Mediterranean climate with hot, dry summers and cool, wet winters with infrequent but regular snowfalls at high altitudes. The winter rainfall is derived from frontal systems that have a strong orographic component which leads to a spatially and temporally predictable rainfall pattern. This stands in contrast to the summer rainfall areas of South Africa in which much of the rain is derived from thunderstorms which are less predictable and less extensive (Westoby 1980 in Le Maitre & Midgley 1992). In the south-western Cape, the heat of the summers is frequently accompanied by persistent prevailing south-easterly winds. Occasionally the prevailing pattern is interrupted by bergwinds which are gusty, hot, desiccating, north-westerly to north-easterly winds that blow from the arid interior across the coastal mountains onto the coast (Geldenhuys 1994). Both wind patterns increase evaporation especially over low-lying areas by

increasing wind speed. In the case of the bergwinds there is also an increase in temperature and decrease in humidity through adiabatic heating as the winds descend from the mountains (Preston-Whyte & Tyson 1988;Geldenhuys 1994). Bergwinds increase the risk of fire (Geldenhuys 1994) in the fire-prone fynbos. The desiccating conditions are only mitigated by orographic fog on the summits of the mountains adjacent to the cold Benguela current of the west coast (Badenhorst 1990; Badenhorst *et al.* 1992). These dramatic seasonal differences have placed strong selective pressure on the organisms living in this biome.

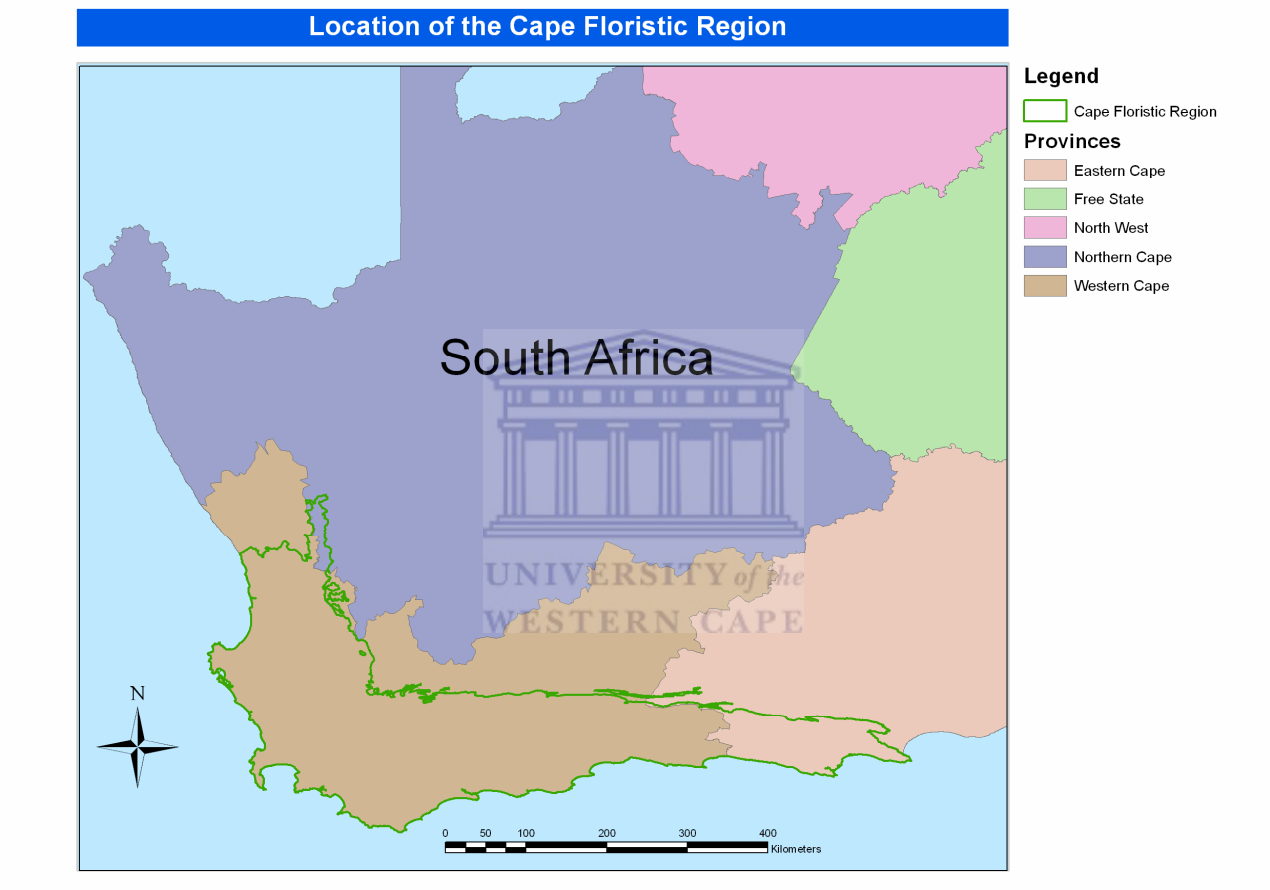

Figure 1. The location and extent of the Cape Floral Region.

1.4 Geology

The Western Cape is dominated by the Cape Fold Mountain belt which runs both east-west parallel to the southern coast and north-south parallel to the western coast. There is a narrow coastal plain between these mountains and the Atlantic Ocean on the west and the Indian Ocean to the south and east. Inland of the mountain cordon there is an escarpment that leads onto the elevated interior. These land forms were originally created by fault rifting of the initially high elevation surface of the southern African area during the break up of Gondwana (Partridge & Maud 1987).

The Cape Fold Mountains (CFM) were formed from deep sedimentary layers deposited from the Ordovician to the Carboniferous periods (500 to 330 ma) in the Agulhas Sea (Hendey 1983a; McCarthy & Rubidge 2005). The origin of the CFM was roughly concurrent with the estimated split between the Lissamphibia and amniote branches at ~338 Ma (Ruta *et al.* 2003). The layers were folded below the surface and thrust up between 278 and 223 Ma (Hälbich 1992). Thus the Cape Fold Mountains were in existence before the divergence of the major neobatrachian lineages from the mid Jurassic to the early Cretaceous 108-202 Ma (Biju & Bossuyt 2003). However, the Cape Fold Mountains were partially overlain by more recent sediments (Dingle 1973; Cowling *et al.* 2009) and have subsequently weathered and eroded to form a relatively low (less than 2500 m) set of dissected and fragmented mountain ranges (see Figure 2). This erosion created the current situation in which nutrient-poor quartzitic mountains from the Table Mountain and Witteberg groups are separated by valleys and lowlands of argillaceous (clayey) soils derived from the more erodible Bokkeveld group and the older (Precambrian) Malmesbury rocks. Two large-scale erosion cycles (African and Post-African I) reduced the majority southern African landscape around the CFM to vast planation surfaces of low relief leaving the CFM, the Namaqua highlands and the Lesotho highlands as upland remnants (Partridge *et al.* 1995). There are very few amphibian fossil sites in the CFR: Langebaanweg (Van Dijk 2003), Duinefontein 2 (Sampson 2003) and Klasies River (Van Dijk 2006) and the latter two sites represent only rather recent (mid- to late Pleistocene epoch) deposits. Unfortunately there are also very few, if any, reliably dated geological features to give an indication of the rate of exhumation of the CFM from the over-lying sediments (M.J de Wit pers. comm.).

The CFM had their orogeny in a set of folding and thrusting episodes between 230 and 278 Ma (Hälbich 1992) or between 223 and 294 Ma (Gresse *et al.* 1992). The first of these sedimentary layers is the Table Mountain Group (TMG) of sandstones and particularly the quartz arenites in the Peninsula Formation. The TMG is overlain by finer sediments (shales) of the Bokkeveld Group (there is also some quartz arenite in the youngest layer (Shone & Booth 2005)). The Bokkeveld Group in turn was overlain by the Witteberg Group which consists of layers of sandstones (largely quartz arenites), mudstones and shales (Broquet 1992). There are several granite intrusions into the Cape Fold Mountains and these resistant rocks remain today e.g. Paarl Rock. Since the orogeny of the CFM, erosion has reduced the height of the CFM and has left a general pattern of more resistant quartzitic sandstone peaks with shales in the intervening valleys. The multiple cycles of deposition, erosion, uplift and folding have left a very complex pattern of mountain ranges and valleys.

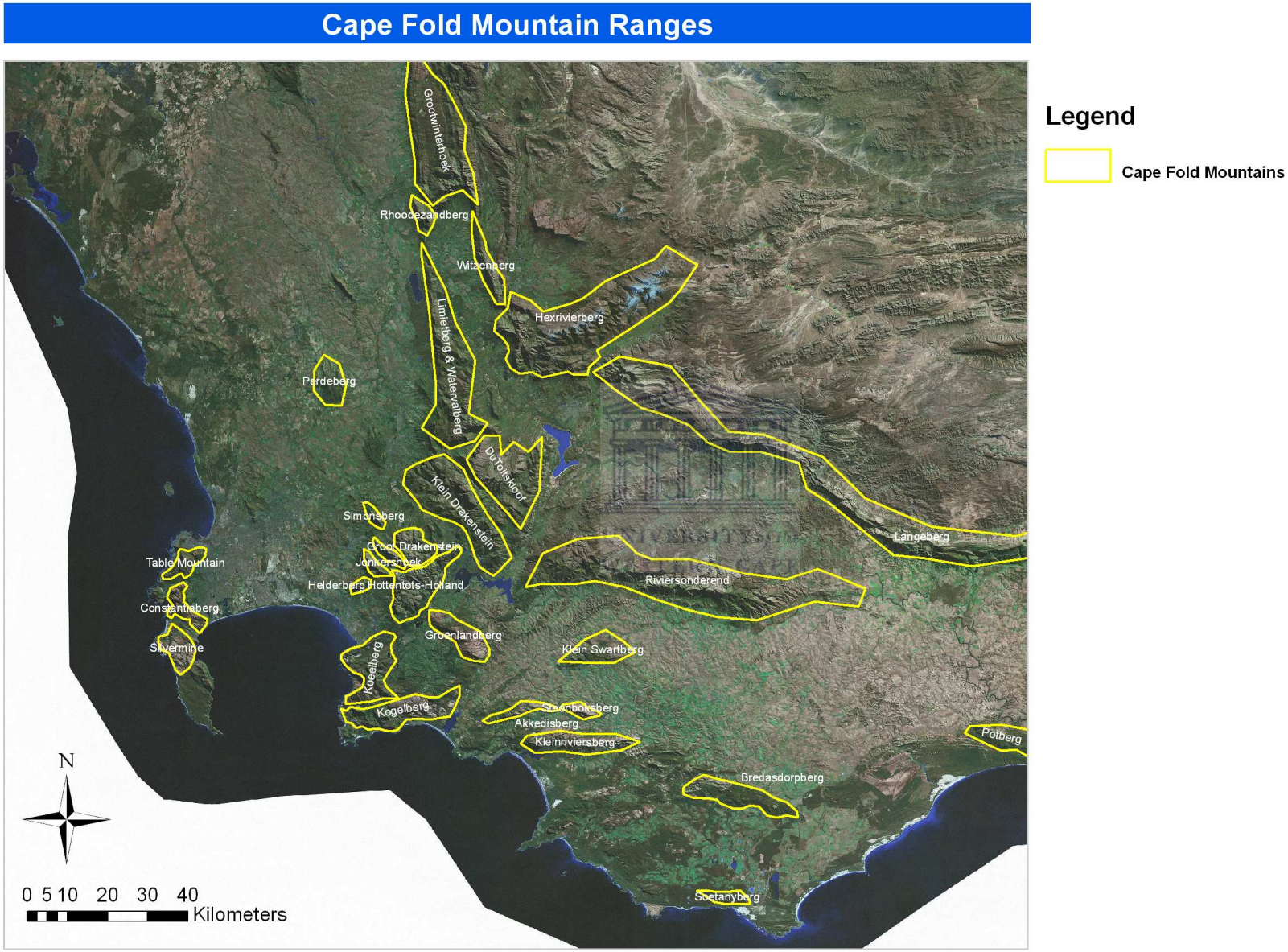

Figure 2. The mountains of the Cape Fold Mountain complex. See Appendix 4 for locations of sampled localities.

1.5 Soils

The arenaceous sandstones of the CFM weather to form quartzitic sands that are highly oligotrophic (low phosphorus and nitrogen contents) (Mitchell *et al.* 1984; Stock & Allsopp 1992). This in combination with the winter rainfall and dry summer climate (see below) are primarily implicated in the evolution of the unique fynbos flora that has evolved and speciated spectacularly in the CFR (see Linder 2003 for a review of the floral radiation). The surrounding argillaceous clays derived from the shales, primarily of the Malmesbury group, contain more nutrients but have a much higher affinity for water and can hold moisture very well at a molecular level. This means that unless these soils are saturated, moisture is unavailable to animals living on these soils. The hot, dry and windy summer months make the clay soils extremely dry, especially at the surface-level.

1.6 Climate

Sea levels were higher in the early Miocene and the climate on the coastal plain was warmer and wetter than present. The coastal areas were well-vegetated with tropical species such as palms as indicated by palynological evidence from the Langebaanweg fossil site (Coetzee & Rogers 1982). The climate in the mountains at this time is not known but can also be assumed to have been tropical i.e. warmer and wetter than at present. In the late Miocene (~10 Ma) the Benguela upwelling system was established and reached its modern extent about 3 Ma (Hendey 1983a). Cold-water upwelling reduces moisture content of the wind blowing off the sea and hence rainfall as this air moves over the land. This effect was strengthened by the uplift of the interior that occurred at roughly the same time, as it blocked the passage of moist air from the Indian Ocean that may have previously been able to reach the western CFM. This causes the Mediterranean climate still experienced in the WCP.

The climate and associated vegetation have also changed over the evolution of the CFM. During the breakup of Gondwana from 140 Ma the climate was humid and subtropical as evidenced by fossil remains of tall gymnosperms and ferns (Cowling & Richardson 1995). This vegetation was replaced by angiosperms starting about 113 Ma which covered the CFR in moist, subtropical forests of Gondwanan and central African species. Between 65 and 35 Ma the climate was warm and wet and was still uniformly covered in forest with a much higher sea level which inundated much of the current coastal lowlands. Roughly 35 Ma the climate dried and cooled leading to a woodland vegetation type containing many fynbos elements. The region had much lower topographic and edaphic diversity in the Oligocene than at present (Cowling *et al.* 2009).

The cold Antarctic Circumpolar Current (ACC) developed as South America broke away from Antarctica with an onset possibly as early as 41 Ma (Scher & Martin 2006) and was in place by 35.5 Ma at the latest (Barker *et al.* 2007). By 34-32 Ma Antarctica was glaciated (Zachos & Kump 2005). This lead to a change in the climate of the CFR as it dried out and cooled down as it was exposed to the ACC. Increased phosphogenesis in the offshore Cape Canyon indicates the onset of warm-water upwelling between 26.1 - 23.6 Ma (Wigley & Compton 2006). There was moderate uplift in the Cape at the end of the early Miocene (~18 Ma, Partridge & Maud 1987) with approximately 200 m of uplift in the east and 150 m in the west. This would have exposed more quartzite and sandstone bedrock and some of the granite intrusions in the west. This new cycle of erosion exposed fine-structured rocks and the clay soils that result from their weathering for the first time. Simultaneously, the deep hydromorphic soils of the major river valleys were replaced by shallow, loamy soils (Cowling *et al.* 2009).

About 12 Ma the Benguela current was in place and further cooling and drying occurred in the CFR generally and on the west coast in particular. By 11-10 Ma there was sustained cold-water upwelling of the Benguela current (Siesser 1980; Kastanja *et al.* 2006; Krammer *et al.* 2006) although there are indications that there was at least intermittent cold-water upwelling in place 15.7 Ma (Wigley & Compton 2006) to 13 Ma (Udeze & Oboh-Ikuenobe 2005). This current has had a profound impact on the climate of the South Western Cape and is largely responsible for coastal temperature and humidity regimes.

There was another cycle of uplift about 2.5 Ma with another approximately 100 m rise in the west and a 200 m rise in the east (Partridge & Maud 1987) adding to the east-west altitudinal asymmetry (Cowling *et al.* 2009). This caused significant incision and resulted in much larger areas of clay soils on the lowlands and steep rocky slopes and cliffs in the mountains (Cowling *et al.* 2009). The uplift events would have had an impact both on the climate of the uplifted land (less moderate and drier inland of escarpment) and increased erosion and down-cutting on the sea-ward side of the escarpment.

In the last 5 million years the climate continued to cool and the typical Mediterranean climate with wet winters and dry summers evolved and the vegetation became dominated by fynbos. Although there were more grazing antelopes and water-dependent large mammals on the coastal lowlands than at present, Luyt *et al.*(2000) suggest that the winter rainfall regime persisted over the middle to late Pleistocene on the west coast of the Western Cape and that an extended rainfall period may have been responsible for supporting these water and grass dependent animals rather than a summer

rainfall regime. Fire had become a regular feature of the fynbos region by the early Pliocene (5 Ma) (Hendey 1983b). Fire remains an important and recurrent feature of fynbos to this day.

Climatic fluctuations in the Holocene were minor. Climatic shifts in the Pleistocene were much larger (Markgraf *et al.* 1995). Fossil evidence indicates that the climate in the western CFR during this period was wetter, cooler and windier than at present with the southern regions colder and drier (Barrable *et al.* 2002). There were higher soil-moisture conditions at Elands Bay Cave (Cowling *et al.* 1999) and evidence of *Podocarpus* forest on the Cape Flats (Schalke 1973) during the last glacial period (10-40 ka). The increased winter rain (decreased summer rain in the eastern CFR) may have lead to the northward expansion of the fynbos in this period. During glacial maxima the western CFR had higher moisture availability (Scott & Woodborne 2007) which is an important feature of potential climatic refugia for amphibians.

To put these variations in climate over the last 20 million years in a global context, they were substantially less than the contemporaneous fluctuations in the northern hemisphere in which extensive glaciation occurred during colder periods (Hewitt 2001; Provan & Bennet 2008). This relative stability has also been suggested as one of the factors that has lead to the massive floral speciation in the CFR (Cowling *et al.* 2009) and may have led to low extinction rates (Barraclough 2006).

1.7 Sea level

Changes in sea level are capable of fragmenting existing habitats or creating new habitats and are thus important to consider to understand biogeographic patterns. Sea levels fluctuated from 150 m above current sea level in the mid Miocene on the west coast to 20 m a.s.l. in the late Pliocene (Hendey 1983c). These transgressions would have flooded much of the coastal plains which then would have been unavailable as habitat for amphibians. Sea level changes are depicted in Figure 3 from Wigley & Compton (2006). Noteworthy are the mid Miocene and Pliocene transgressions from about 16 Ma to 12 Ma and 5 Ma to 3.5 Ma respectively.

The retreat to modern levels is dated to 3.5-2 Ma (Wigley & Compton 2006). During the Quaternary sea levels did not rise more than about 6 m above the current sea level but fell as much as 120 m exposing a much larger expanse of coastal plain (Hendey 1983a). Conversely the mountains have been relatively stable and their form relatively unchanged through the Cenozoic (Hendey 1983c).

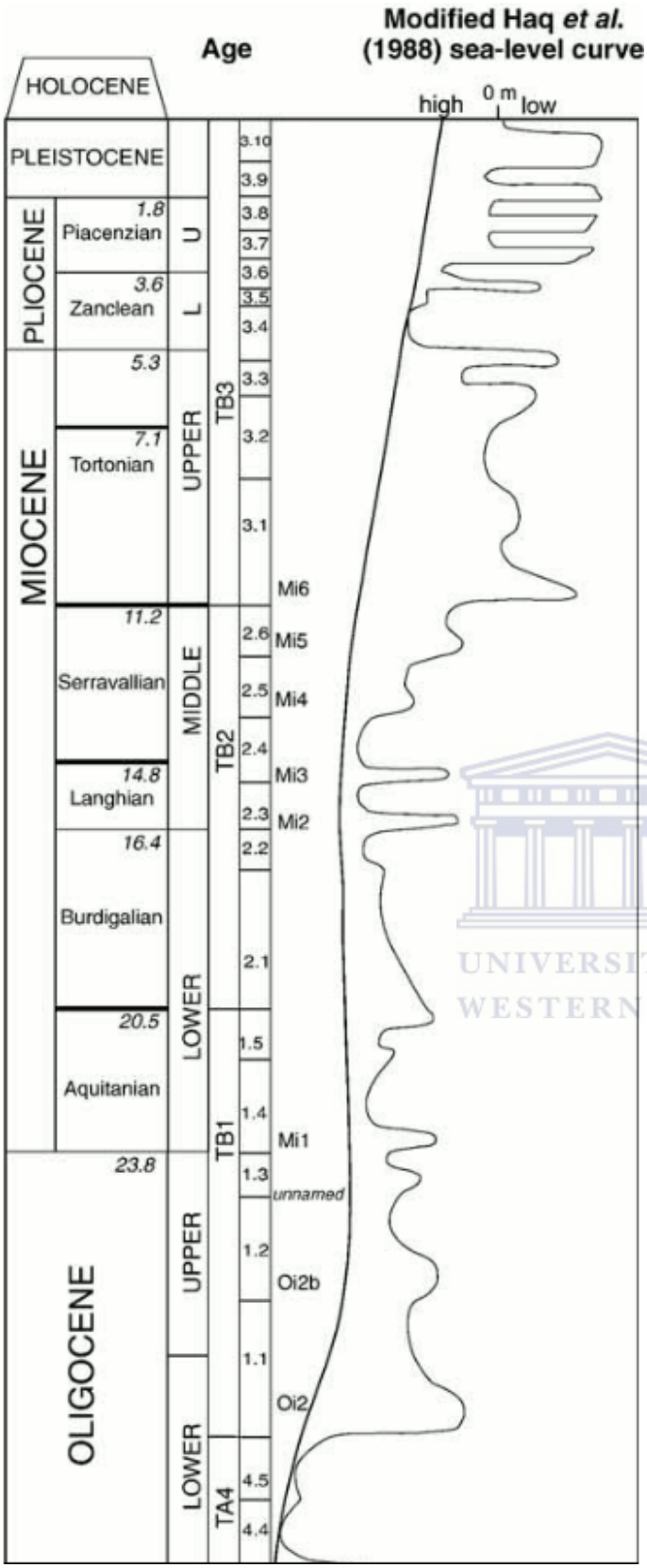

Figure 3. Cenozoic sea level changes along the western margin of southern Africa (modified from Wigley & Compton 2006).

1.8 Vegetation

The fynbos vegetation is superimposed on the complex topography of the Cape Fold Mountains (CFM). This complex topography has been suggested as one of the reasons for the extraordinary diversity of the fynbos biome by providing a multitude of niches for specialists (Lock 1978; Linder 1985; Cowling & Lombard 2002; Linder 2003; Losos & Glor 2003; Linder & Hardy 2004; Linder 2005; Lips *et al.* 2008; Cowling *et al.* 2009). It must be noted that floral species richness is not uniform within the CFR (Cowling & Lombard 2002) and the differing patterns in the east and the west of the CFR are discussed in Chapter 4. There are also dramatic differences in climate between the western and eastern CFR (Cowling & Lombard 2002; Chase & Meadows 2007). The western CFR has a strong winter-wet rainfall pattern whilst rainfall in the eastern CFR is less predictable, lower and spread throughout the year. The eastern parts of the CFR do still receive some rainfall from the Indian Ocean during the summer months in addition to the frontal rainfall in the winter months. The geographic break between the summer-dry CFM and the all-year rainfall area is at the Caledon and Riviersonderend mountain ranges (Figure 4; Chase & Meadows 2007).

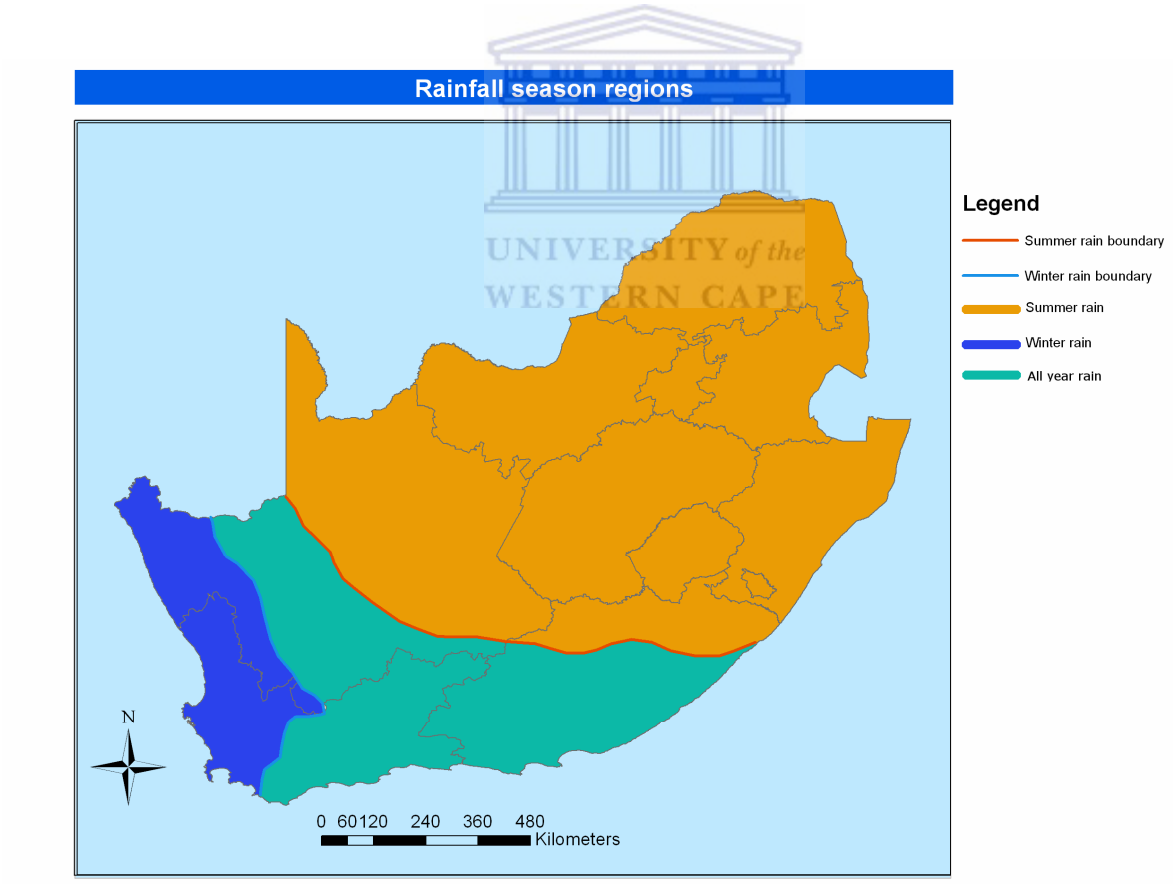

Figure 4. Distribution of rainfall seasonality across the Western Cape Province, after Chase & Meadows (2007).

The changes in plant species and consequently the vegetation communities they form have been related to the changes in climate that have occurred in South Africa (Cowling & Lombard 2002;

Midgley *et al.* 2001)). Changing climates and vegetation types have also been postulated to have driven speciation in various other taxa in the CFR: in dwarf chameleons, genus *Bradypodion* (Tolley *et al*. 2004; Tolley *et al*. 2006); in agamid lizards, genus *Agama* (Swart *et al.* 2009); in lacertid lizards, genus *Pedioplanis* (Makokha *et al.* 2007). There has been a recent synopsis of the processes that form and maintain diversity in the CFR that broadened the focus beyond plants (Linder *et al.* 2010) but did not cover the amphibian diversity of this region.

The implications of this temporal and spatial complexity for cladogenesis and speciation of *Arthroleptella* are dealt with in Chapter 3 on phylogeography.

1.9 Systematic history

Hewitt (1926) described the genus *Arthroleptella* as distinct from *Arthroleptis* (Smith) based on the entire omosternum in *Arthroleptis* which is also smaller than the cartilaginous metasternum in *Arthroleptella.* The type species of the genus is *Arthroleptella lightfooti,* originally described as *Arthroleptis lightfooti* by Boulenger (1910), based on a single specimen (presumably a female based on the white ventral colouration) from Newlands on the Cape Peninsula (Boulenger 1910). Hewitt describes *Arthroleptella* as having a very indistinct tympanum in contrast to the distinct tympana of *Arthroleptis* (Hewitt 1926). This is in contrast to Boulenger's description in which he records the tympanum as distinct. In the same publication Hewitt describes *Arthroleptella bicolor* which he distinguishes from *A. lightfooti* on the basis of the shape of the xiphisternum which is bifid in *A. bicolor* but anchor-shaped in *A. lightfooti* and that *A. bicolor* is larger and more robust.

Hewitt (1935) described *A. bicolor villiersi* from Jonkershoek in the Hottentots-Holland Mountains based on:

- it being a stout form like *A. bicolor* but having simple digit tips as opposed to the slightly enlarged digit tips in *A. bicolor*;
- the snout more pointed in *A. lightfooti*;
- *lightfooti* always having two distinct metatarsal tubercles with the disparity in size between the two metatarsal tubercles rather more pronounced in *A. villiersi*;
- the breadth of the interorbital space, sub-equal to or not much exceeding the breadth of the upper eyelid in *A. lightfooti*, 1.4 times or even nearly twice the breadth of upper eyelid in *A. villiersi*: the eye being relatively larger in *A. lightfooti*;
- a xiphisternum which is "sometimes notched mesially but not bifid - apparently broader than in A. *bicolor* or A. *lightfooti*".

Deckert (1938) described *Arthroleptella ahli* from Lichtenstein in the Eastern Cape Province of South Africa. Loveridge (1957) synonymised this taxon with *Rana delalandii delalandii* (Duméril & Bibron) as *Arthroleptis ahli*. Poynton (1964) synonymised *Arthroleptella ahli* with *Tomopterna cryptotis* based on the obvious morphological differences between the two genera.

Fitzsimons (1947) described *Arthroleptella hewitti* from the Drakensberg as differing from *A. lightfooti* and *A. bicolor* by its large size, colour markings, poor development of subarticular tubercles and absence of palmar, plantar and inner metatarsal tubercles, and also external vocal sacs (in males); the 4th finger in *A. hewitti* extending well beyond the distal subarticular tubercle of 3rd (in *A. lightfooti* and *A. bicolor* not further than distal tubercle of 3rd); a cartilaginous precoracoid in *A. hewitti* as opposed to the bony precoracoids in *A. lightfooti* and *A. bicolor*.

Fitzsimons (1947) described *Arthroleptella hewitti minor* from Bulwer as differing from *A. hewitti* by being smaller, less robust, hind limb and fifth toe proportionately shorter and a minute outer metatarsal tubercle present.

Poynton (1964) placed *Arthroleptella* in the subfamily Cacosterninae and stated that *Anhydrophryne* can be derived from *Arthroleptella*. He also stated that *Arthroleptella* and *Anhydrophryne* together can be derived from a 'primitive *Phrynobatrachus* stock' (Poynton 1964). To test this hypothesis, the origin of the genus *Arthroleptella* will be explored by constructing a genus-level phylogeny.

Poynton (1964) defined the genus *Arthroleptella* as having the following characters: Pupil horizontal; procoracoid-clavicular bar not or only partially ossified along anterior margin; omosternum ossified; metasternum ossified, slender, but not exceeding length of coracoid symphysis; vomerine teeth absent; a smaller inner, and sometimes an outer, metatarsal tubercle present; subarticular tubercles on hands and feet poorly developed; webbing absent.

Poynton (1964) synonymised *A. bicolor* and *A. bicolor villiersi* with *A. lightfooti* based on greater variation in the shape of the xiphisternum, size, colouration, size of eye, shape of snout and swellings on digital tips in the greater number of specimens available for measurement. This statement was made on the basis of the examination of 63 specimens from Table Mountain, Muizenberg Mountains , Wellington, Jonkershoek, Assegaaibos, Koëlbaai and the Hermanus Mountains. Poynton (1964) also synonymised *A. hewitti minor* and *A. lawrencei* (Loveridge) with *A. hewitti.*

Description of the various forms of *Arthroleptella* up to this point had been based entirely on morphological characters although (Hewitt 1926) did note that Walter Rose had described the call as a "very high-pitched chirp, like that of a cricket".

Bishop & Passmore (1993) described *A. ngongoniensis* from the KwaZulu-Natal mist belt. It was described on the basis of unossified procoracoids, ventral margin of coracoids entire, outer metatarsal tubercle absent, the absence of webbing, pupil horizontal, entire omosternum unossified, immaculate venter with the throats of both males and females pale yellow and terrestrial breeding behaviour. In 1994, Channing *et al.* described *A. drewesii* from the Kleinrivier Mountains near Hermanus. It was distinguished based on its long call duration and cytochrome b gene sequence differences. In this paper they recognized *A. villiersi* and *A. bicolor* as full species in addition to *A. hewitti, A. ngongoniensis and A. lightfooti.* The elevation of the previously synonymised *A. bicolor* and *A. villiersi* was supported based on call and cytochrome b fragment sequence differences. This was the first paper since 1947 to ascribe *Arthroleptella* populations in the Western Cape to species other than *A. lightfooti*. This was also the first paper to examine the calls from different *Arthroleptella* populations and use this character to distinguish species.

They listed *A. bicolor* as occurring to the west of the Wellington Du Toits and Riviersonderend mountains; *A. drewesii* on the Kleinrivier Mountains; *A. lightfooti* on Table Mountain and other slopes on the Cape Peninsula; and *A. villiersi* in the Palmiet River valley, Hottentots-Holland, Jonkershoek and Swartboskloof mountains. They state that not all mountains have been surveyed and that a "detailed field study is essential to determine the ranges of these species". The current study is a direct response to this appeal.

In 2000, Dawood and Channing described *A. landdrosia* from the Hottentots-Holland Mountains and present the first phylogeny of the genus *Arthroleptella* based on molecular sequence data from a 442 kb fragment of the 12S mitochondrial gene. *Arthroleptella landdrosia* was distinguished based on its smooth dorsal skin, widely spaced nostrils and a call duration longer than *A. drewesii*. It was noted that the species is sympatric with *A. villiersi.*

Turner *et al*. 2004 described *A. subvoce* from the Groot Winterhoek Mountains of the Western Cape Province near Porterville (not to be confused with the Groot Winterhoek Mountains of the Eastern Cape near Uitenhage). It was shown to be the sister species to *A. bicolor*.

Van der Meijden *et al.* (2005) noted that *Pyxicephalus, Tomopterna, Natalobatrachus, Amietia (*as *Afrana), Cacosternum* and *Strongylopus* formed a well supported southern African ranid clade and noted that the other African Cacosternine genera *Arthroleptella, Microbatrachella, Poyntonia* and *Nothophryne* would lead to an even stronger diversity hotspot in South Africa. A similar strongly supported clade was reported by Bossuyt *et al.* (2006) comprising *Pyxicephalus, Tomopterna, Poyntonia, Cacosternum, Arthroleptella, Natalobatrachus, Amietia (*as *Afrana)* and *Strongylopus.* Bossuyt *et al.* (2006) also noted the presence within this clade of a monophyletic "Cape Province clade" comprising *Arthroleptella, Natalobatrachus, Poyntonia* and *Cacosternum.*

In a combined morphological and mitochondrial fragment analysis Scott (2005) showed that *Arthroleptella hewitti* formed a sister group with *Anhydrophryne rattrayi* rendering *Arthroleptella* paraphyletic. Scott (2005) suggested that *Arthroleptella* be placed in the Cacosterninae as a subfamily of the Ranidae along with *Cacosternum, Ericabatrachus, Nothophryne, Microbatrachella, Poyntonia* and *Anhydrophryne*.

Frost *et al.*'s 2006 expansive phylogenetic assessment of global amphibian diversity placed *Arthroleptella* in the newly erected family Pyxicephalidae containing *Amietia (*including *Afrana), Anhydrophryne, Arthroleptella, Aubria, Cacosternum, Natalobatrachus, Pyxicephalus, Strongylopus* and *Tomopterna*. The genera *Microbatrachella, Nothophryne,* and *Poyntonia* were provisionally placed in the same family. This arrangement agrees well with the findings of Van der Meijden *et al.* (2005).

Dawood and Stam (2006) reviewed the status of the frog genus *Anhydrophryne* which was proposed as a close relative of *Arthroleptella* by Poynton (1964) and Scott (2005). Based on a phylogeny using 12S and 16S mitochondrial gene fragments Dawood and Stam (2006) recommended transferring *Arthroleptella hewitti* and *Arthroleptella ngongoniensis* to *Anhydrophryne*. This arrangement resulted in three species in *Anhydrophryne* (*A. hewitti*, A. *ngongoniensis* and *A. rattrayi*) and six species in *Arthroleptella (A. bicolor, A. drewesii, A. landdrosia , A. lightfooti, A. subvoce* and *A. villiersi).* This arrangement resulted in a clear separation of the Western Cape species in *Arthroleptella* from the Eastern Cape and KwaZulu-Natal species in *Anhydrophryne*.

Turner and Channing (2008) described *Arthroleptella rugosa* from the Klein Swartberg Mountains near Caledon based on 16S mitochondrial gene sequences, irregular skin and very distinctive calls. They also presented a phylogeny describing the position of *A. rugosa* relative to the previously described species of *Arthroleptella* which placed it as sister to *A. lightfooti* and *A. villiersi*.

Turner & Channing (in prep) describe three new species of *Arthroleptella* from the Western Cape. This last publication brings the current state of taxonomic and systematic research into *Arthroleptella* up to date and forms the basis for the taxa examined in this thesis.

1.10 Species concepts

The identification of species boundaries is set by the species concept that one employs. Thus it is critical in a systematic account to clearly state the species concept that is used. Unfortunately species concepts have long been a contentious issue in biology and remain so. I think it sufficient here to very briefly discuss species concepts in relation to the particular species concept I have adopted.

Changes in advertisement calls have long been hypothesized to play a role in speciation as the coupling of male advertisement calls with female mate choice maintains distinct genetic populations e.g. (Blair 1955; 1958; 1960; Littlejohn 1960; Gerhardt & Schwartz 2001). This coupling of signalling and receiver systems forms the basis of the recognition concept as defined by Paterson (1985). Under this concept a specific mate recognition system (SMRS) evolves to facilitate sexual reproduction. The generalisation of Paterson's recognition concept to other forms of reproduction will not be pursued here. In the case of frog advertisement calls a signal evolves to attract individuals of the opposite sex which recognize the call and allows the potential mate to find the calling individual. In most frogs it is males that utter the advertisement calls and females use phonotaxis to locate the calling male (Blair & Littlejohn 1960), particularly in prolonged breeders (Wells 1977). A feature that plays such a central role in facilitating reproduction will also be central in the evolution of populations and the process of speciation. If there is a shift in the signal (advertisement call) that diverges beyond the bounds of conspecific recognition then those individuals producing the divergent signals should be strongly selected against as they will not attract mates. It is this feature that leads to Paterson's thesis of stabilising selection on the SMRS.

I have not applied the Biological Species Concept (Mayr 1942; 2000) or the Recognition Concept (Paterson 1985) despite parts of these concepts having some utility. These concepts are not useful for understanding speciation in *Arthroleptella* due, apart from other theoretical failings such as relying on indirect evidence for gene flow and shared genealogy, to the difficulty of assessing either isolating mechanisms or recognition in this group. The Evolutionary Species Concept (Wiley & Mayden 2000) is conceptually appealing and internally consistent but is operationally uninstructive as taxa can be delimited at any level.

Various concepts, definitions and operational approaches have been developed under the umbrella term of the Phylogenetic Species Concept e.g. (Meier & Willmann 2000; Mishler & Theriot 2000; Wheeler & Platnick 2000). There has been varying emphasis placed on the meaning and use of monophyly to delimit valid taxonomic groupings and whether ancestral species survive speciation (for example see Meier & Willmann 2000, Mishler & Theriot 2000, Wheeler & Platnick 2000). Monophyly provides a good indicator that lineages are evolving separately but there are exceptions to this pattern (see Funk & Omland 2003). For example, in the early stages of speciation, incomplete lineage sorting may yield polyphyly or paraphyly.

Vences & Wake (2007) provide a lengthy review of species concepts and species boundaries in the context of phylogeographic studies of amphibians. They propose that there is agreement on the goal of species concepts to gain an understanding of when evolutionary lineages have irretrievably diverged. Similarly, De Queiroz (2007) aims to consolidate the common ground in many of the species concepts and does so by explicitly distinguishing between the operational aspects (species delimitation) from the conceptual aspects of species concepts. He has only a single necessary requirement for a species *viz*. that it constitutes a separately evolving metapopulation lineage. He terms this the Unified Species Concept.

Speciation is a process and we see it at different stages in its progression. This means that depending on the time over which a population or set of populations is tracked the resolution and associated certainty with which we assign species distinctions varies. This is well illustrated in Figure 5 taken from De Queiroz (2007). In the ‘Gray Zone’ individuals in the left and right evolutionary pathways are likely to be very similar so morphological, genetic or behavioural differences may not reflect the speciation event. The divergence depicted in this diagram could also have been shown to be temporary with the two branches coalescing again if the genomes were still compatible in which case it could be argued that speciation did not occur.

One of the problems faced by taxonomists and systematists is that the future of evolutionary branches is unknown and that there are typically only a few reference points from which to construct the evolutionary trees (phylograms) with modern samples necessarily dominating the evidence. Constructing hypotheses based on such evidence as is available is the only rational route of progress. Sites & Marshall (2003, p. 468), note that “there is agreement that speciation processes create ‘fuzzy’ boundaries under which all methods will occasionally fail or be discordant with each other”. These hypotheses can be tested as better evidence and techniques are developed.

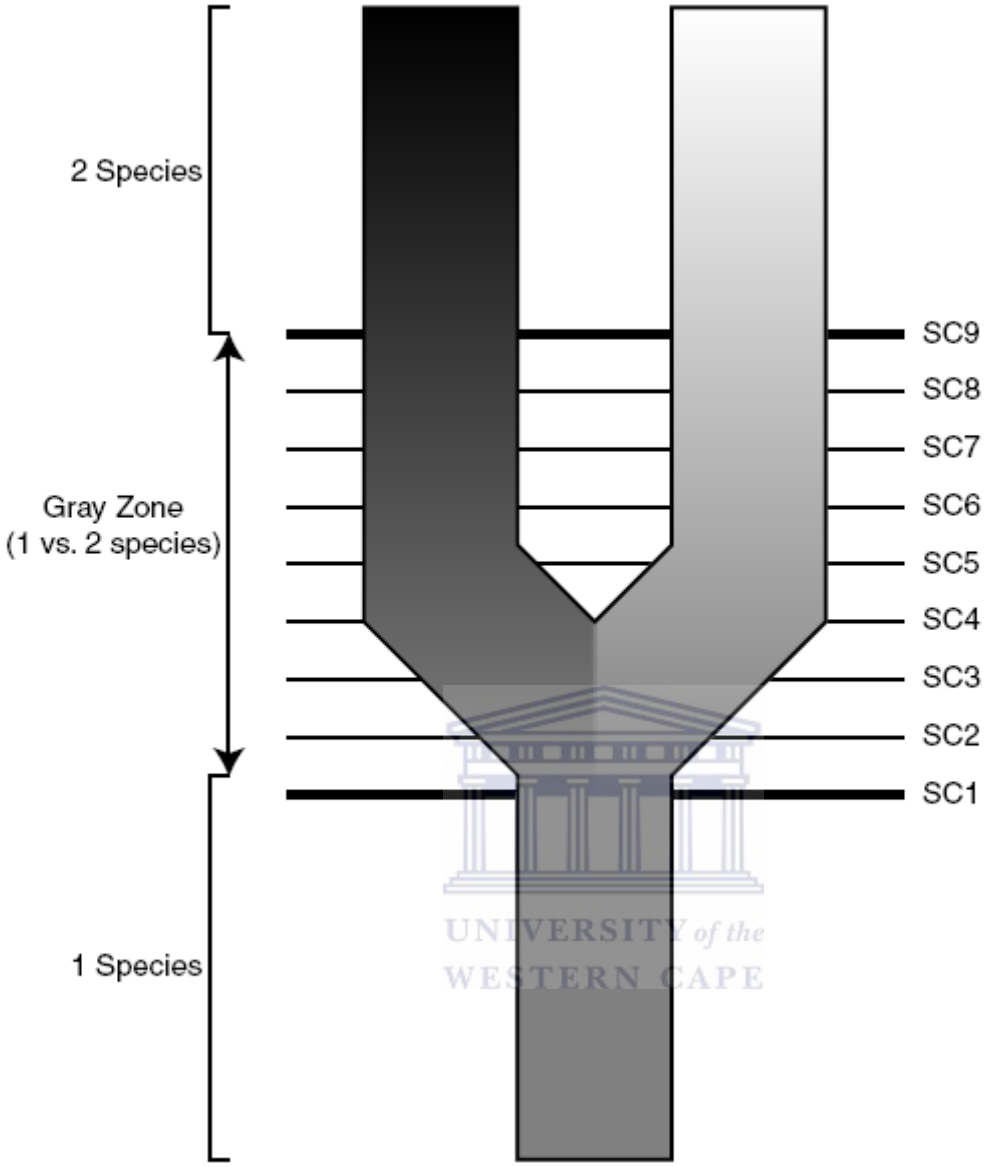

Figure 5. Diagram depiction speciation over time on vertical axis from SC1 to SC9 (from de Queiroz 2007).

Species are not equivalent entities despite the assertion by Cunningham & Cherry (2000) that they should be treated as equivalent. It is now clear that speciation and the associated genetic divergence happens at different rates in different organisms (Rand 1994; Hoegg *et al.* 2004; Sims *et al.* 2009). Indeed, there is little reason to expect that different species will evolve at the same rate even within a genus. This is because the life history traits of individual species affect effective generation time, effective population size, sex ratios and several behavioural factors that may determine the rate at which genetic change occurs (see Verheyen *et al.* 1996). Another fundamental influence on the degree of divergence is of course the time since speciation. As speciation events for many species, even closely related species, are not necessarily contemporaneous, the degree of sequence

divergence between species pairs will not be equivalent. This is contrary to the approach taken by Cunningham & Cherry (2000) in which genetic distance is used to infer a consistent application of species boundaries. Some species boundaries are easy to observe, others are not, depending on our insights and knowledge of the organisms involved. For example if one knows that mating is preceded by advertisement calls and selective phonotaxis then these auditory characters may easily be detected by humans and tested as diagnostic species characters. Greater difficulties may arise in species in which we do not know what the behaviourial and physiological mechanisms are that facilitate gene transfer.

The species concept used here is that a species is a separately evolving set of metapopulations that share genes through sexual reproduction and generally do not actively share genes with other metapopulations. They are thus are genetically differentiated and have been evolving separately for a long period of time i.e. more than 10 000 years. This figure is used as speciation is known to have occurred within this time frame (Hoskin *et al*. 2005). New species arise primarily through allopatric speciation where populations become too isolated to share genes frequently enough to avoid genetic drift affecting key reproductive and other characters and so end up on differing evolutionary trajectories. All populations isolated for long enough will speciate (through genetic drift or other more directed forms of evolution) or go extinct. This concept is compatible with de Queiroz's Unified Species Concept (De Queiroz 2007) in which species are viewed as separately evolving metapopulation lineages. The evidence required to show that metapopulations are evolving separately can take many forms although evidence of genetic sequence divergence is arguably the most direct consequence of separate evolution. If additional evidence is gathered, for example from phenetic or behavioural data that supports the genetic pattern, then the genetic data are corroborated. If conflicting data are obtained, they must be explained so that the final assessment is free of contradiction as an evolutionary explanation.

1.11 Research aims, questions and hypothesis tests

In this study I aim to build a comprehensive phylogeny of the genus *Arthroleptella* and to answer the following questions:

How is the genus *Arthroleptella* related to other frog genera?

When and where did the genus *Arthroleptella* evolve?

How many species are there in the genus *Arthroleptella*?

How are these species related to each other?

What are the distinguishing characters of each species?

This thesis also aims to review of the systematics of *Arthroleptella* and to provide an evolutionary context for the pattern of speciation in this genus using phylogeographic methods. This will answer the following questions:

Which evolutionary processes gave rise to the species in *Arthroleptella*?

Where did the speciation events take place?

When did the speciation events take place?

The predicted stabilising effect on advertisement calls leads to the following hypotheses to explain variation between different populations:

1) Calls will be very similar (not significantly different) between geographically close populations.

2) Calls will be very similar (not significantly different) between closely related or recently diverged populations. The inference being that call structures are inherited and do not diverge easily or quickly from ancestral calls.

3) If there have been long periods of time separating populations, then these populations should show significant differences in advertisement calls as the effects of random genetic drift or directional selection cause the calls to diverge as the stabilising selection between populaiton that are isolated is released.

The following hypotheses apply to advertisement calls, genetic sequences and morphology:

H_0 = There are no significant differences between *Arthroleptella* populations. The statement of this null hypothesis may seem extreme or biologically unlikely but as several previous works (Poynton 1964; Passmore & Carruthers 1979; Passmore & Carruthers 1995) have regarded all Western Cape populations of *Arthroleptella* as a single species *viz. Arthroleptella lightfooti* it is appropriate to formally test this hypothesis.

Alternative hypotheses are based on developing biologically sound models of evolution for this genus based on biogeography and population genetics. The alternative hypotheses that will be tested in this thesis are:

H_1 = There are significant differences in between *Arthroleptella* populations from different (geographically isolated) mountain ranges.

H_2 = There are significant differences between all *Arthroleptella* populations.

H_3 = There are significant differences between *Arthroleptella* populations with different overall call structure types.

Plus H_{02}, an additional null hypothesis that there are no significant differences within populations from the currently described species. This is to be tested against the following alternative hypothesis:

H_4 = There are significant differences between *Arthroleptella* populations from within currently described species.

Within these hypotheses, pairwise comparisons allow the identification of the source of the significant differences i.e. the particular populations or species that cause the difference.

Another aim is to test the Channing *et al.* (1994) hypothesis of morphological similarity and to establish if certain morphological characters do have systematic value and if so, to what extent. These tests will inform the taxonomic arrangement of species which is still unsatisfactory. A related aim is to establish what the distribution of each species is. Once the distributions have been established then steps can be taken to investigate what the threat status of each species is.

1.11.1 Hypothesis testing approach

I have chosen to conduct some population comparisons in a traditional null hypothesis significance testing framework as an initial step in describing patterns across space and to assess their magnitude in a well-known analytical framework. It is noted that this approach to a complex biological dataset with relatively small sample sizes is liable to be insensitive to differences that may be biologically meaningful, particularly with alpha set to 0.05 or less. I have set alpha at 0.05 as I have no prior reason to deviate from the traditional approach which provides comparable results to published work. However, if the application of null hypothesis significance testing consistently rejects the null hypotheses for the variables tested above, it provides a strong indication that the patterns of difference are not random effects and explanations should be sought that are the result of directed processes. Information theoretic based techniques will then be well suited to building models to explain these patterns. The maximum parsimony (MP) and Bayesian approaches to phylogeny generation and the use of Akaike's information criterion in evolutionary model choice (see Methods below) go some way to advance this approach. The call and morphological data still require the attention of information theoretic approaches but this falls beyond the scope of this thesis.

1.11.2 Thesis structure

Chapter 2 describes the methods used for obtaining and analysing the distribution, advertisement call, genetic sequence and morphological data are described.

Chapter 3 shows the spatial distribution of *Arthroleptella* and presents the results of the advertisement call analyses and statistical tests; the genetic sequences analyses are presented as a set of phylogenetic trees and networks and tests of gene flow; the morphological measurements and morphometric ratios are presented. The available information to inform conservation assessments is also presented in this chapter.

In Chapter 4, the geographical distribution of *Arthroleptella* and the concomitant variation in advertisement calls, phylogenetic patterns and morphological measurements are discussed. The role that barriers to gene flow and variations in advertisement calls may have in the process of speciation in this group is also discussed.

Chapter 2. Methods

2.1 General approach

The study of evolution and speciation of moss frogs was informed by the investigation of the relationships between extant species through the formal techniques of systematics. These relationships are typically represented by cladograms, phylograms and networks (the latter capable of revealing reticulate relationships (Bandelt *et al.* 1995). These procedures rely on either genotypic or phenotypic evidence or both (Wheeler *et al*. 2006). Genotypic evidence has the advantage of being a representation (as a sequence of nucleotides) of the very material that is passed directly from ancestor to descendant. However, there are only four nucleotides. Each of these are completely interchangeable i.e. an adenosine base in a particular position inherited from one ancestor cannot be distinguished from an adenosine based inherited from a different ancestor at the same site. It is therefore impossible to test hypotheses of nucleotide homology in isolation and only tests of character congruence can be applied to them (Wheeler *et al*. 2006). On the other hand, each piece of phenotypic evidence can be tested for inferences from homology although phenotypic evidence may suffer from effects that are not heritable e.g. developmental or environmental effects. These effects will be discussed below as they pertain to the phenotypic characters under investigation in this study.

2.2 Distribution

The distribution of *Arthroleptella* was assessed by field visits to all of the major mountain ranges of the CFR. Sites that were visited were georeferenced using a Garmin e-trex Vista handheld GPS. Historical occurrences as stored in the CapeNature Biodiversity Database were used where these had been georeferenced and checked for georeferencing errors. These data were overlain on vector and orthorectified image layers to check for spatial relationships using ArcView 3.2 and ArcGis 9.3 (ESRI). The following layers were used:
geology (ENPAT /TOURPAT, Department of Environment Affairs and Tourism), vegetation types (Mucina & Rutherford 2006) and Landsat and SPOT 5 imagery.

To investigate the biogeographical history of the region in which moss frogs have evolved the distribution of frog species in the eastern and western CFR was compared. This was done by using occurrence records from the CapeNature Biodiversity Database which includes the results of the South African Frog Atlas Project (Minter *et al*. 2004) and the latest available field guide to the region (Du Preez & Carruthers 2009).

2.2.1 Call recordings

Calls of male frogs were recorded in the field. Most recordings were obtained using a Marantz PMD 670 solid state digital recorder and Audio-Technics AT897 microphone. Older recordings (prior to 2004) were made on a Sony MZR55 Mini-Disc recorder with an AKG directional microphone. Most air temperatures during the call recordings were measured using a Fluke 51II thermometer. Some of the older temperatures were measured with a MC Systems 620 digital thermometer and occasionally a Oregon Scientific THGR228N thermometer-hygrometer was used as a backup when batteries failed in the Fluke thermometer. Differences in the readings of all three thermometers were less than 1°C (difference between MCS & Fluke 0.9°C, difference between Fluke and Oregon Scientific 0.2°C at 20°C).

2.3 Call analysis

Analogue calls from tape recordings and Mini-Discs were digitised into (.wav) format (Lin16 encoding at 41 000Hz sampling rate) using a SoundBlaster Audigy 2 sound card on a PC. Call recordings originally made on a digital recorder were not modified.

Calls were analysed using WaveSurfer 1.8.3 sound analysis software (Sjölander 2005). Calls were measured using the time and frequency measurement tools in WaveSurfer whilst examining both a spectrogram and wave form representation of the sound. For each call, the number of notes, number of pulses per note, duration, dominant frequency and note interval were measured.

The effects of temperature on all these variables was tested for each species separately as well as for the genus as a whole.

Calls were tested for statistically significant differences in the number of notes per call, number of pulses per call, number of pulses per note and dominant frequency by means of ANOVA with Bonferroni *post hoc* tests. Measures of central tendency in anuran acoustic signals such as means are robust where sample sizes are large (Littlejohn 2001). Call and morphological analyses were conducted using SYSTAT version 11 (Systat Software) and R version 2.8 (R Development Core Team 2009).

2.4 Molecular analysis

Tissue samples were taken from the thigh muscle or from toe clips. DNA was extracted using Chelex polymer beads, standard phenol chloroform extraction methods (Palumbi *et al*. 1991; Palumbi 1996) or modified chloroform extraction methods in which the phenol:chloroform step is

omitted. The concentration of the extracted DNA was measured using a fluorometer. The gene fragments, primers for amplification and the PCR protocols used for amplification are listed in Table 1. The choice of genes used for phylogenetic reconstruction was based largely on those fragments that had been successfully used in the literature for similar purposes and to provide a range of rates of evolution to allow assessment of both relatively recent as well as older divergences. For capturing more recent divergences the mitochondrial 12S and 16S fragments were used, e.g. (Goebel *et al.* 1999; Dawood & Channing 2000; Hertwig *et al.* 2004; Vences *et al.* 2004; Lehr *et al.* 2005; Hoskin 2004; Hillis & Wilcox 2005; Vences *et al.* 2005). For capturing deeper phylogenetic patterns several coding nuclear gene fragments were amplified (Bossuyt & Milinkovitch 2000; Vences *et al.* 2003; Hoegg *et al.* 2004).

Table 1. Genes used in this study with primers, PCR protocols.

		Primer pairs	PCR protocol
Mitochondrial	12S	12SJ & 12SK (Goebel *et al.* 1999), t-Phe & t-val (Wiens *et al.* 2005).	2 min at 96ºC, 33-35 cycles of: 30 sec at 94ºC, 45 sec at 52ºC and 90 sec at 72ºC; 7 min at 72ºC.
	16S	16Sar-L and 16Sbr-H (Palumbi *et al.* 1991).	2 min at 94ºC, 33-35 cycles of: 30 sec at 94ºC, 30 sec at 56ºC and 60 sec at 72ºC; 5-7 min at 72ºC.
Nuclear	Rag-1	Amp-Rag1F & Amp-RAG1R1, Amp-RAG1F1 & Amp-RAG1R (San Mauro *et al.* 2004)	2 min at 94ºC, 35-39 cycles of: 30 sec at 94ºC, 45 sec at 48ºC and 90 sec at 68ºC or 72ºC; 7 min at 68 or 72ºC.
	Rag-2	Rag2A.F35 & Rag2.Lung.320R (Hoegg *et al.* 2004)	2 min at 94ºC, 35-39 cycles of: 30 sec at 94ºC, 45 sec at 48ºC and 90 sec at 68ºC; 7 min at 68ºC.
	Rhodopsin	Rhod-1a & Rhod-1d (Bossuyt & Milinkovitch 2000)	2 min at 96ºC, 35 cycles of: 30 sec at 94C, 45 sec at 55ºC and 60 sec at 72ºC; 7 min at 72ºC.
	Tyrosinase precursor	Tyr1C & Tyr1G (Bossuyt & Milinkovitch 2000)	2 min at 96ºC, 35 cycles of: 30 sec at 94ºC, 45 sec at 55C and 60 sec at 72ºC; 7 min at 72ºC.

PCR reaction volumes were made up to 25 µl with 12.5 µl Kappa Taq or Go Taq Ready Made Master Mix (both 2x concentration), 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), 3-4 µl of sample, 1 µl of BSA (1mg/ml) and 6-5 – 5.5 µl purified water.

The gene fragments were sequenced by the Stellenbosch University Central Analytical Facility using an ABI 3730xl sequencer. Sequences were examined for signal strength and ambiguous base pairs were corrected where it was obvious that they had been incorrectly assigned based on reverse primer sequences and signal strength. Sequences with poor signal strength were re-amplified and re-sequenced. Sequences were aligned with published sequences to make sure that the sequenced products matched the gene fragments targeted by the PCR. Sequences were aligned using Clustal X 1.81 (Chenna *et al.* 2003) and Clustal X2 (Larkin *et al.* 2007).

2.5 Phylogenetic analysis

2.5.1 Phylogenetic tree reconstructions

Relationships between individuals were examined through the use of three phylogenetic tree construction methods. The primary method was Bayesian analysis conducted using Mr Bayes (Ronquist & Huelsenbeck 2003). This method formally incorporates the use of prior information about the distribution of characters and likelihoods of character changes and uses the maximum likelihood function which is known to outperform other phylogenetic estimation methods (Huelsenbeck *et al.* 2002). This method was preferred to true maximum likelihood methods as it computationally much more efficient and because of the explicit incorporation of prior information. In addition, Hobbs & Hilborn (2006) point out that the application of Bayes Law produces probabilities of hypotheses. This property seems well suited to testing of phylogenetic reconstructions. The Bayesian approach explicitly deals with phylogenetic and mapping uncertainty (Ronquist 2004). It does not make sense to ignore what we know about nucleotide evolution when nucleotide distributions are the very basis for phylogenetic reconstructions using genetic sequences. A similar point is made more generally by Hobbs & Hilborn (2006) and Stephens *et al.* (2007) in that progress in science, and in particular ecology and evolution, will be best served if we use prior information. Bayesian approaches with varying and flexible models of nucleotide evolution can cope with DNA sequences evolving under substantially different substitution rates and patterns which may give erroneous results with older phylogenetic reconstruction methods (Edwards 2009).

Mr Bayes settings:
Inverse gamma rates
2 million generations
Burnin 2000 generations
Convergence was taken to have occurred when the average standard deviation between two runs had declined to below 1% as suggested in (Ronquist *et al.* 2005).

Secondly, parsimony analysis was conducted to examine the relationships as represented by the most parsimonious path of character change using PAUP 4b10 (Swofford 1998).

PAUP settings:
Heuristic maximum parsimony searches were conducted in PAUP with 1000 bootstrap replicates as a conservative assessment of clade accuracy (Hillis & Bull 1993). The setting used for these searches were constructed as follows:
Hsearch addseq=random nreps=1000 rearrlimit=5000000 limitperrep = yes
Bootstrap nreps=1000 brlens=yes rearrlimit=5000000 limitperrep=yes
Maxtrees was set to autoincrement by 100 whenever the maximum number was encountered.

A third approach using direct optimisation as implemented in POY (Wheeler *et al*. 2006) does not require a prior alignment using a method such as that employed in Clustal-X. Direct optimisation simultaneously aligns and constructs a phylogram using dynamic homology e.g. (Wheeler 2006; Wheeler *et al.* 2006). This approach is useful for dealing with large-scale sequence re-arrangements.

POY settings:
set(root:"Tomopterna")
build (1000)
swap ()
select ()

These methods were compared to ascertain whether there were contradictions in the topology of the relationships retrieved and how the phylograms related to other information gathered, in particular the geographic arrangement of genetic and other patterns. Methods that result in phylogenies that could be squared with the other evidence and inference were considered less well supported. The use of multiple methods enables an evidence-based approach to evaluate them.

Another commonly used technique for generating phylogenies is maximum likelihood (Holder & Lewis 2003). This method was not used for the purely operational reason that it is computationally very time consuming (e.g. Holder & Lewis 2003).

2.5.2 Placement of *Arthroleptella* in the Pyxicephalidae

To properly evaluate the status and relationships of species within the genus *Arthroleptella* it is necessary to place the genus in an evolutionary context. To this end a family level phylogeny was constructed to establish which of the pyxicepahlid genera is closest to *Arthroleptella*. This is necessary to allow inferences to be made about the ancestral distribution and habitat preferences in addition to genetic, call and other characters. The genera *Rana* and *Phrynobatrachus* were chosen as outgroup taxa based on the broad taxonomic sampling by Frost *et al*. 2006 which placed the Phrynobatrachidae as sister to the Pyxicephalidae and *Rana* as the type representative of the family Ranidae which was the family in which the pyxicephalid genera were previously placed (Dubois 2003).

2.5.3 Model testing

To allow examination and comparison of the information contained in the different gene fragment sequences, each fragment was used separately to construct single-fragment phylograms in Mr Bayes. All gene fragment sequences were combined in partitioned data sets to examine the pattern produced by the entire suite of genetic sequence evidence whilst still allowing the modelling of the evolution of each gene fragment to be treated independently (Ronquist & Huelsenbeck 2003). Models of nucleotide evolution were evaluated for each gene fragment using jModelTest 0.1.1 (Posada & Crandall 1998; Posada & Crandall 2001; Posada 2008). This procedure makes use of the Akaike Information Criterion corrected for small samples sizes (AICc) (Burnham & Anderson 2004; Posada & Buckley 2004) to evaluate model fit which is recommended as a model choice criterion (Posada & Buckley 2004; Sullivan & Joyce 2005) as opposed to likelihood ratio tests (Posada & Crandall 1998; Posada & Crandall 2001). Results of these tests were used to inform the models of nucleotide substitution for the generation of Mr Bayes phylograms.

2.5.4 Phylogenetic network reconstructions

Graphical depictions of the relationships between samples were constructed using median joining networks (MJ) (Bandelt *et al.* 1999) with the Steiner tree (Polzin & Daneshmand 2003) cleanup option to remove unnecessary median vectors as implemented in Network 4.5 (Fluxus Technology Ltd. 2009). Median joining networks seem more robust to missing data than other network joining methods such as statistical parsimony and minimum spanning networks (Joly *et al.* 2007).

2.6 Phylogeography

2.6.1 Identification of population and species boundaries

To investigate the spatial arrangement of genetic patterns several tests for significant spatial patterns of genetic variation and visual displays of these patterns were carried out. The techniques chosen did not require prior assignment of individuals to populations. The approach was rather to use the patterns of genetic variation to inform decisions as to where population boundaries may lie (e.g. Manel *et al.* 2003; Holderegger & Wagner 2008) and then evaluate these patterns in an evolutionary and biogeographically framework. To investigate these patterns a number of different tests were conducted.

Population differentiation was examined using analysis of molecular variation (AMOVA), a derivation of ANOVA specifically for genetic data using F statistics (Excoffier 1992). The AMOVA population comparisons and population differentiation tests were conducted using the functions in Arlequin 3.11 (Excoffier *et al.* 2005). Identification of populations was based on both results of the phylogenies and biogeographic groupings. Only 16S sequence data were used for this procedure as it was the gene with the best sample size both in absolute terms (79 georeferenced individuals) and in the number of spatial localities sampled (50 sites).

AMOVAs were conducted with various different groupings of sequences into populations depending on the proposed species hypotheses. Analyses were conducted by grouping individuals according to the following groups:

First all populations were placed into a group comprising *A. lightfooti, A. rugosa and A. villiersi;* and a group comprising *A. sp.* A, *A. bicolor, A. drewesii, A. sp.* B, *A. landdrosia,* and *A. subvoce* based on the phylogeny presented in Turner & Channing (2008). These two groups correspond to basic differences in the structure of the advertisement call. The group containing *A. lightfooti, A. rugosa and A. villiersi* is termed the Chirping clade and the other group the Clicking clade. A separate set of analyses was conducted with just the Clicking clade in which populations were grouped as either belonging to the Landdrosia clade (*A. sp.* A, *A. drewesii, A. sp.* B, *A. landdrosia*) or the Bicolor clade (*A. bicolor and A. subvoce*). Within the Landdrosia clade, *A. sp.* A was further split into *A. sp.* A East and *A. sp.* A West groups. The western group was represented by specimens from Amanzi and Jonaskop and the eastern group by specimens from Die Galg, Kanonberg and Twistniet. In addition, *A. landdrosia* was also split into *A. landdrosia* and *A. landdrosia* "Houwhoek" groups. The *A. landdrosia* "Houwhoek" group refers to specimens form Houwhoek and Groenlandberg mountains.

Population differentiation was further examined in a more spatially explicit manner using spatial analysis of molecular variance (SAMOVA). This was performed using SAMOVA 1.0 (Dupanloup *et al.* 2002). Mantel tests, genetic landscape shapes, and Monmonier algorithm analyses were conducted using Alleles In Space (Miller 2005). Mantel tests allow the testing of association between two matrices, originally of spatial and a temporal (Mantel 1967) measures, and in this application for testing the association between genetic distance and geographic distance. Genetic landscape shapes as implemented in Alleles In Space (AIS) provides a spatially explicit visualisation of the distribution of genetic variability over the landscape. AIS does this by interpolating genetic distances and then spatially plotting residual genetic variation (to account for spatial correlation with genetic distances). The Monmonier algorithm identifies the spatial location of disjunctions in genetic sequences. It can be applied sequentially to reveal progressively smaller disjunctions.

The 16S mitochondrial fragment was used for all these tests of the relationship between geographic distance and genetic distance to maximize spatial sampling as more 16S sequences from a larger number of different sample sites were available. Positions containing ambiguities were removed for the Mantel and Monmonier algorithm analyses as these techniques interpret ambiguities as a new state which may be misleading and may thus overestimate genetic distance. Mantel tests, landscape shapes and Monmonier algorithm analyses were conducted using residual genetic distances. Several different 16S data sets were used to test for different spatial patters. Sequence data for all *Arthroleptella* species was used for examining spatial spread of genetic divergence across the entire genus to see if isolation by distance was a general pattern that may explain the evolution of the different species across the genus distribution range. The hypothesis that isolation by distance may be the driving force in the speciation of the genus derives from the general pattern that most species are allopatrically distributed. The null hypotheses in the AMOVA and SAMOVA tests are that the number of groups of populations and groups of populations are not significantly different from each other. Identification of which populations are different is done using various pairwise comparisons between populations.

The spatial pattern of speciation was examined using dispersal-vicariance analysis (DIVA) as implemented in DIVA 1.2 (Ronquist 1997). To carry out this analysis populations were grouped into 15 areas as illustrated in Table 2.

Table 2. Areas used in dispersal-vicariance analysis. See Figure 2 for locations of mountain ranges and Appendix 2 for a detailed listing of locations visited.

Area label	Area
A	Agulhas
B	Du Toitskloof mountains
C	E. Riviersonderend Mountains
D	W. Riviersonderend Mountains
E	Kleinriviersberg
F	Hottentots-Holland and adjacent mountains
G	Caledon Klein Swartberg
H	Peninsula
I	Helderberg
J	Grootwinterhoek Wilderness Area
K	Kogelberg
L	Limietberg
M	Bredasdorpberg
N	Groenlandberg & Houwhoek mountains
O	Groot Drakenstein & Franschhoek mountains

2.7 Timing

To assess the timing of cladogenic events in the evolution of *Arthroleptella* a relaxed clock Bayesian approach was taken using BEAST (Ho *et al.* 2005; Drummond & Rambaut 2007). The phylogenetic trees derived from parsimony analyses were not used in the estimation of speciation events because the exact distribution used to model among-site rate variation has been found to be critical for a successful phylogenetic analysis (Buckley & Cunningham 2002).

BEAST was configured to use a birth-death speciation process. Monophyletic groups were defined as represented in Table 3 below. An uncorrelated relaxed clock was used to estimate the timing of coalescent events. The TN93 substitution model was used for the Rag-1 sequences and the GTR model for the mitochondrial 12S and 16S sequences as indicated by the results of jModelTest.

Age calibrations for these groups were based on estimates of divergence times from Van der Meijden *et al.* (2005), including their confidence intervals to describe a normal distribution about age estimates to address issues of precision about estimated ages (Graur & Martin 2004; Hedges & Kumar 2004).

Table 3. Labels assigned to monophyletic genus groups with mean ages and standard deviations used to calibrate speciation timing events.

Label	Mean age (Ma)	Std. dev. (Ma)	Constituent genera
CS	40.2	8.4	*Cacosternum, Strongylopus*
CSM	47.7	9.3	*Cacosternum, Strongylopus, Amietia*
ANCSM	50.4	9.7	*Arthroleptella, Natalobatrachus, Cacosternum, Strongylopus, Amietia*
ANCSMPHT	61.7	11.3	*Arthroleptella, Natalobatrachus, Cacosternum, Strongylopus, Amietia, Poyntonia, Anhydrophryne*
ANCSMPHTY	69.9	12.3	*Arthroleptella, Natalobatrachus, Cacosternum, Strongylopus, Amietia, Poyntonia, Anhydrophryne, Pyxicephalus*
ANCSMPHTYBR	91.9	14.9	*Arthroleptella, Natalobatrachus, Cacosternum, Strongylopus, Amietia, Poyntonia, Anhydrophryne, Pyxicephalus, Phrynobatrachus, Rana*

2.8 Morphological measurements

2.8.1 External morphology

Specimens were measured using a Wild MMS 235 digital micrometer with a Zeiss stereo microscope. The following measurements were taken:

head-body length (measured from tip of snout to tip of urostyle)
head width (at widest part)
snout to eye distance
inter orbit distance
inter nares distance
eye width
eyelid width
femur length
tibia length
foot length
toe length as measured along longest toe (toe 4)

These measurements were examined individually and as pairwise ratios to assess proportional differences in morphology. The locations of these measurements are shown in Figure 6.

In addition to these quantitative measures, several qualitative characters were also noted during examination of the specimens:
degree of expansion of finger and toe tips; development of inner and outer metatarsal tubercles; dorsal and ventral colouration; and general appearance.

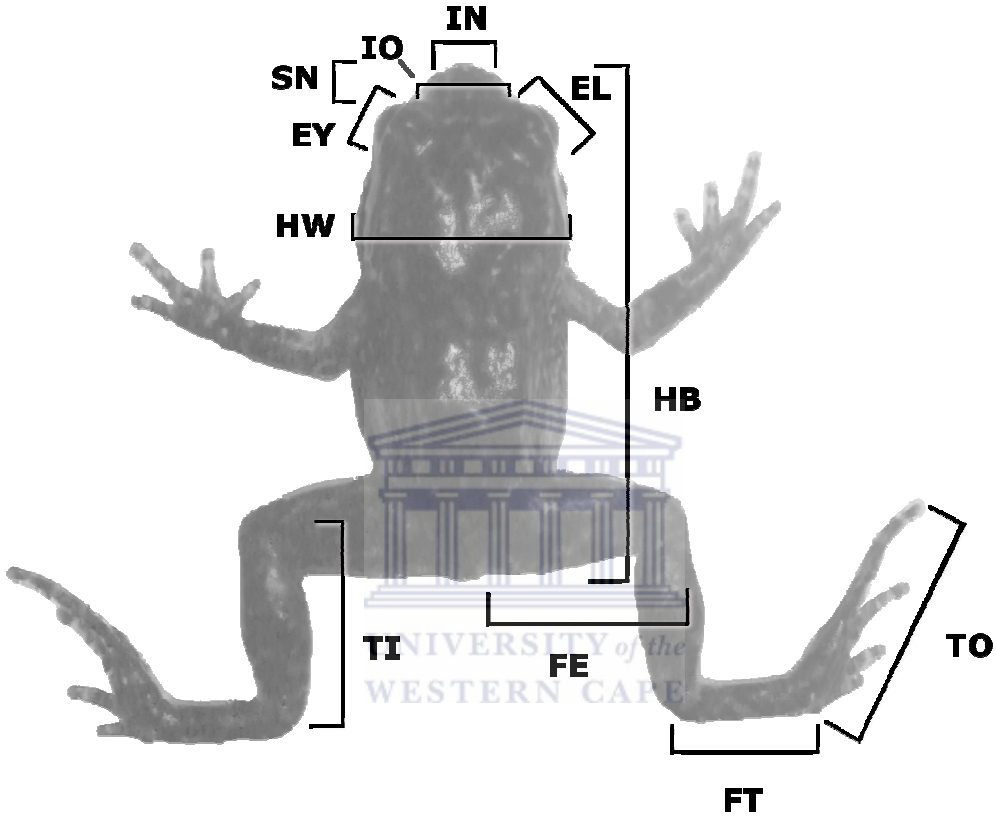

Figure 6. Locations of morphological measurements. Key: EY = eye, SN = snout, IO = inter orbit, IN = inter nares, EL = eyelid, HB = head and body, TO = toe, FT = foot, FE = femur, TI = tibia, HW = head width.

2.8.2 Skeletal morphology

Representative specimens were x-rayed onto colour photographic film to allow examination of the skeletal structures.

2.9 Conservation status

Conservation status was arrived at by assessing the threat of extinction according to the IUCN Red List method (IUCN 2001; 2008). This method uses a set of formal criteria to assign a taxon to a particular extinction risk category viz. Extinct, Extinct in the wild, Critically Endangered, Endangered, Vulnerable, Near Threatened, Least Concern. In cases where the information to make a threat assessment is lacking, the species is classified as Data Deficient. The IUCN Red List of

Threatened Species is regarded as a useful tool for conservation planning, monitoring populations and conservation decision making according to a consistent set of criteria (Rodrigues *et al.* 2006).

Data to address IUCN criterion A – past, present of future reduction in populations was not available for this study. However, in cases where there is loss of habitat, it can be inferred that it will lead to a reduction in population size. However, there are few if any records of *Arthroleptella* previously occurring in areas where they do not occur currently and the level of uncertainty involved in calculating habitat area lost precludes making use of this criterion.

The B criterion is based on the extent of the distribution of a particular taxon and is often the primary informant in assessments of poorly known species as is the case with *Arthroleptella*. Distribution under criterion B is measured by Extent of Occurrence (EOO) and Area of Occupancy (AOO). Extent of Occurrence is a measure of the geographic spread of risk over all populations as the larger this measure is the less likely will be that all populations simultaneously go extinct. Area of Occupancy is the spatial extent that is actually occupied by the species (IUCN 2008; Gaston & Fuller 2009). To apply the B criterion, estimates of Extent of Occurrence and Area of Occupancy were made as follows:
Calculation of EOO was done by creating minimum convex hulls (MCH) and alpha hulls (AH) around known distribution points. An alpha hulls are similar in concept to a MCH but it allows for populations to be split into to more than one polygon if points are so far apart that representing the population as a single polygon exaggerates the area. The threshold at which this distance is exceeded is set by the value of alpha. Alpha was set to 2 to address this possible bias in minimum convex hulls (Burgman & Fox 2003).

Calculation of AOO was done by carrying out the following two steps:
A) potentially suitable habitat was mapped in a geographic information system (ArcView GIS). Suitable habitat was identified by overlaying known distribution points over vegetation types (Mucina & Rutherford 2006), topography (as 20 m contours), rivers and natural colour imagery derived from Landsat 7 image (EarthSat) for the years 2000 and 2002. Unsuitable areas were identified as transformed land (urban areas, industrial areas and agricultural lands), areas dominated by argillaceous soils, sea cliffs and estuaries (which have high salinities). The area of the suitable habitat was then taken to represent AOO. Mapping of habitat was done at a coarse scale of roughly 1 km. This resulted in a set of polygons that delimited the extent of the distributions.

B) AOO was estimated by 10 % of the EOO figure as a rough estimation of the amount of land surface that may constitute suitable habitat. This method is likely to lead to overestimations of AOO. The only way to accurately measure AOO is to map every seep, suitable bog and stream side at a mapping resolution of no coarser than 5 m. Identifying these sites from high resolution imagery is possible but such imagery was not uniformly available for the entire range of the genus and it would be very time consuming to manually map all such sites although large sites are clearly visible in images from the height of the dry season or post-fire. Development of an algorithm to automate mapping at 5 m resolution may be feasible depending on the availability of imagery that can accurately reflect surface water as well as vegetation at 5 m resolution or better.

Estimation of population size is required for application of IUCN criterion C which concerns small population size and continuing declines in population size and for criterion D for very small or restricted populations. For *Arthroleptella* species identified as likely to be of conservation concern it was possible to derive estimates of total population size based on the numbers of calling males. This was done in a simple categorization of population size as follows:

<10

>=10 <20

>=20 <50

>=50 <100

>=100

Criterion E which is based on the results of formal population habitat viability analyses (PHVA) requires many, sufficiently detailed life history parameters that unfortunately are not available for any *Arthroleptella* species and so this criterion was not used in this assessment. However, various life history parameters that do bear on extinction risk in this genus are discussed in the conservation section of the Discussion.

Chapter 3. Results

3.1 Distribution

Searches for moss frogs across the CFM resulted in the presence and absence shown in Figure 7. A total of 192 distribution records of moss frogs was collected in this study. Compared to historical records, these records expanded the distribution of moss frogs northwards from Bainskloof to the Grootwinterhoek mountains and eastwards from Riviersonderend to near Cape Agulhas. Moss frogs were not found north of the Grootwinterhoek mountains or further east than Cape Agulhas. The genus is restricted to south of the Olifants River and to the west of the Breede River. Presence is closely associated with mountains. The distribution of historical *Arthroleptella* presence records is contained within the bounds of distribution found in this study (Figure 8).

There is a strong correlation between the presence of moss frogs and arenites (primarily quartzitic sandstones derived from TMS) as shown in Figure 9. A few populations were found on granite (e.g. Paarl Mountain).

Moss frogs were found to be restricted to permanently wet habitats in Southwest Fynbos bioregion (Mucina & Rutherford 2006, Figure 10) in the Western Cape Province (WCP) of South Africa. In the few cases where moss frogs have been found in forest, these forests are surrounded by fynbos and they occur in the surrounding fynbos too. Moss frogs avoid flowing water and are generally restricted to seeps and marshy areas. These habitats are very patchily distributed in the generally arid conditions of this province (see Figure 11). There may also be habitat choice within seeps. It is noticeable that not all seeps or even all parts of a single seep are suitable for some species. For example *Arthroleptella landdrosia* is restricted to certain parts of a seep area that it shares with *A. villiersi* at Landdroskop. This has been noted before by Dawood & Channing (2000) and they state that *A. landdrosia* only calls from very wet drainages. Similarly *A. subvoce* is restricted to the upper reaches of mountain seeps even though the slopes across the seeps are gentle. The scale of this habitat association is in the order of tens of metres or less.

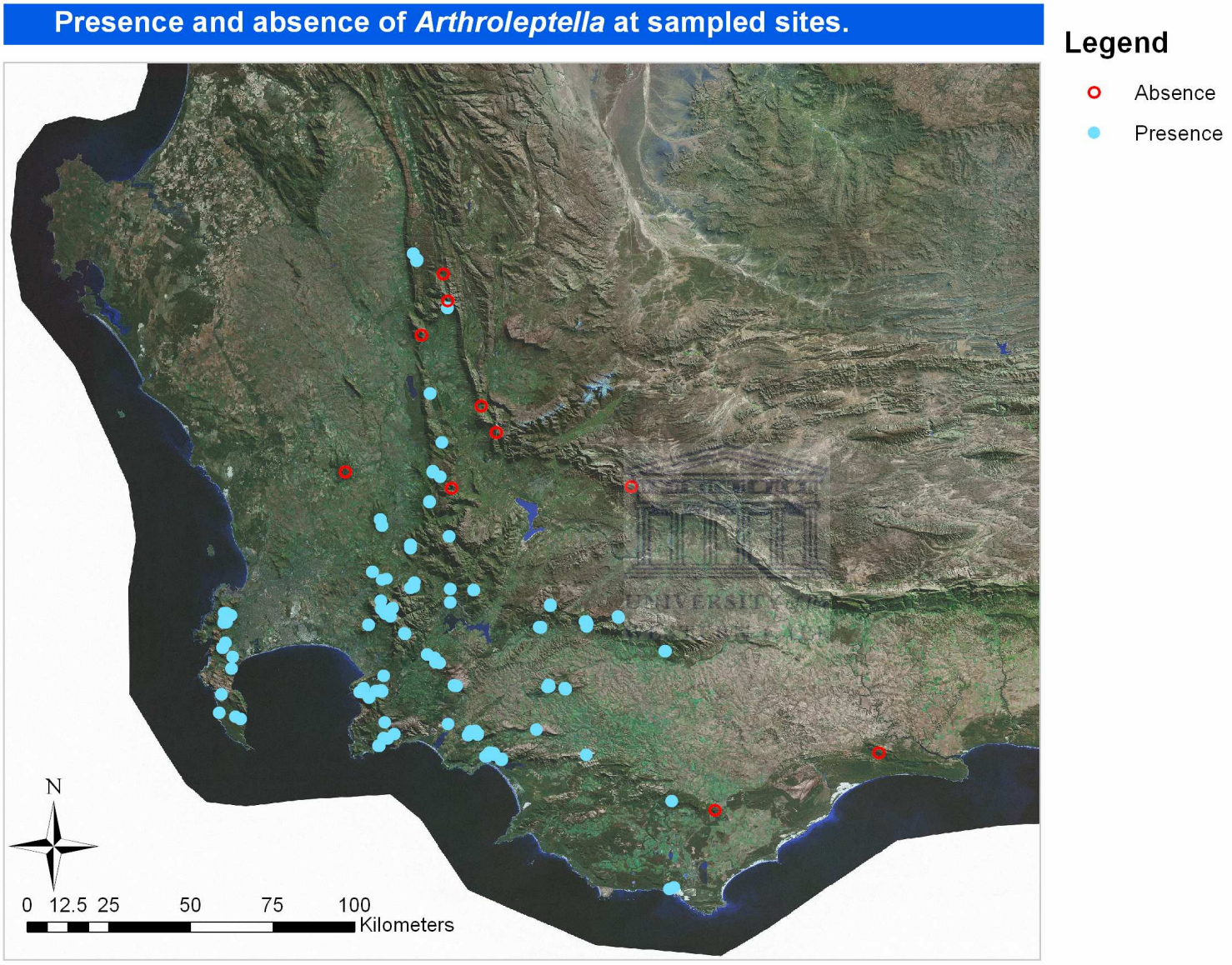

Figure 7. Sampled sites showing presence and absence of *Arthroleptella* recorded in this study.

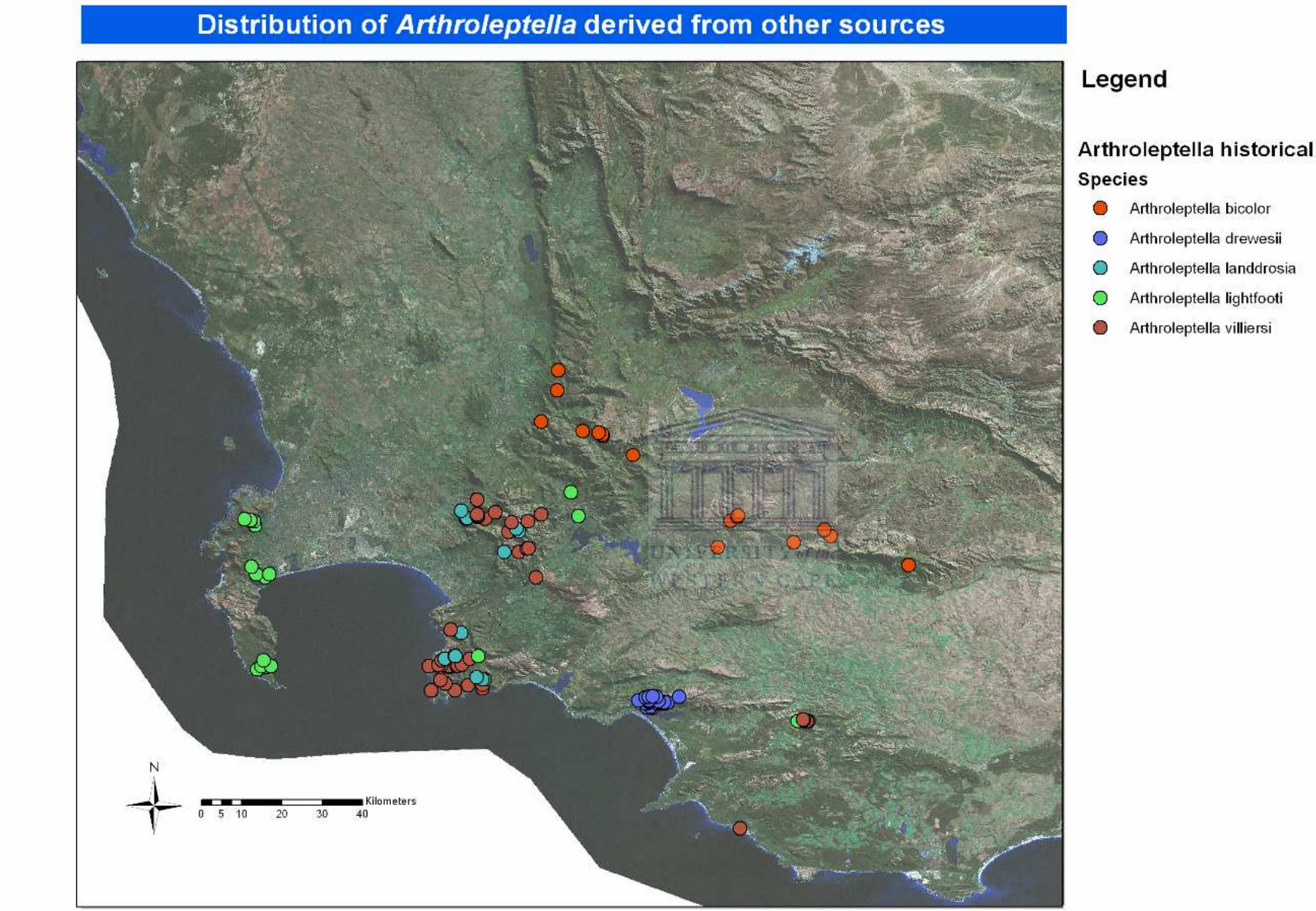

Figure 8. Sampled sites showing recorded presence of *Arthroleptella* from other sources.

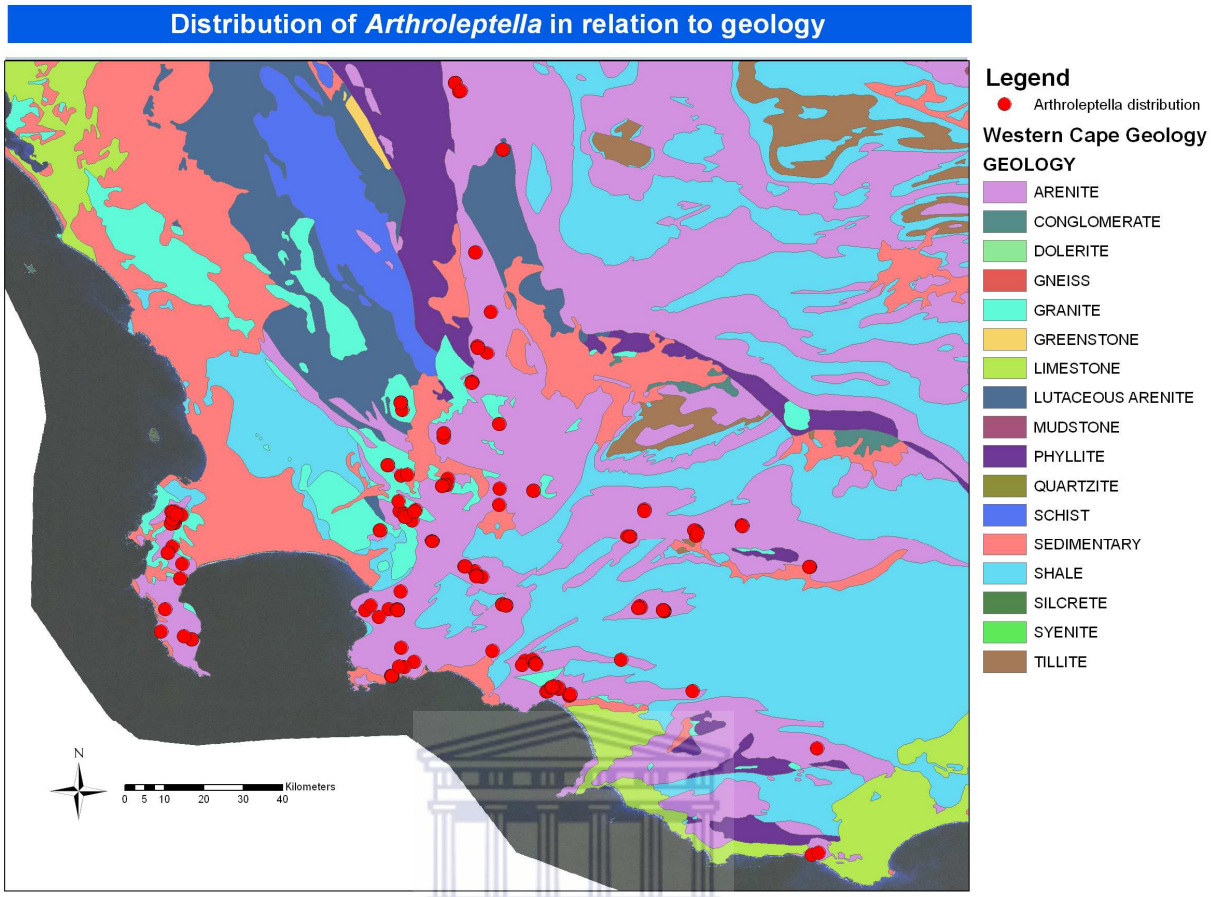

Figure 9. Distribution of *Arthroleptella* superimposed over geology (ENPAT/TOURPAT, Department of Environment Affairs and Tourism)

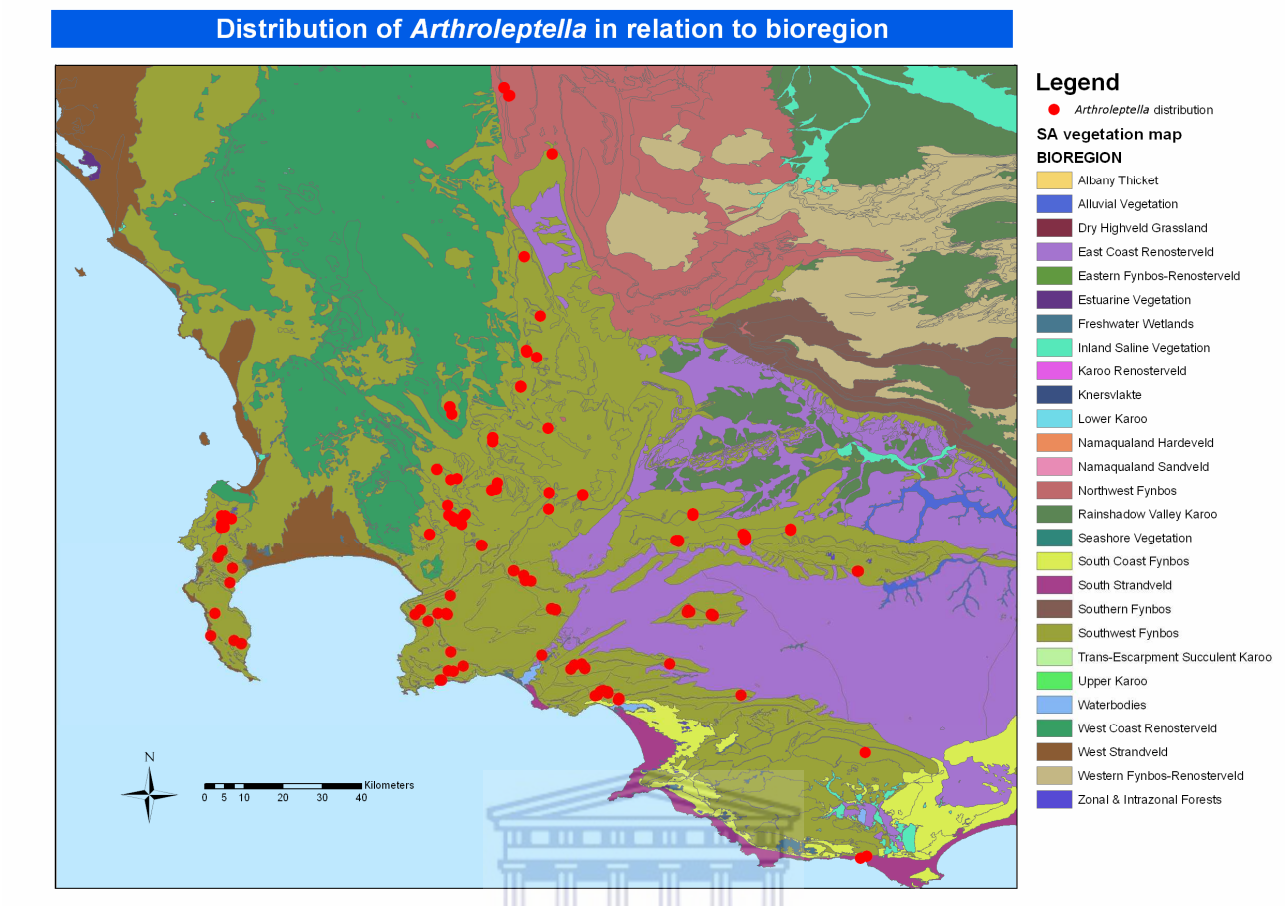

Figure 10. Distribution of *Arthroleptella* superimposed over bioregions (Mucina & Rutherford 2006).

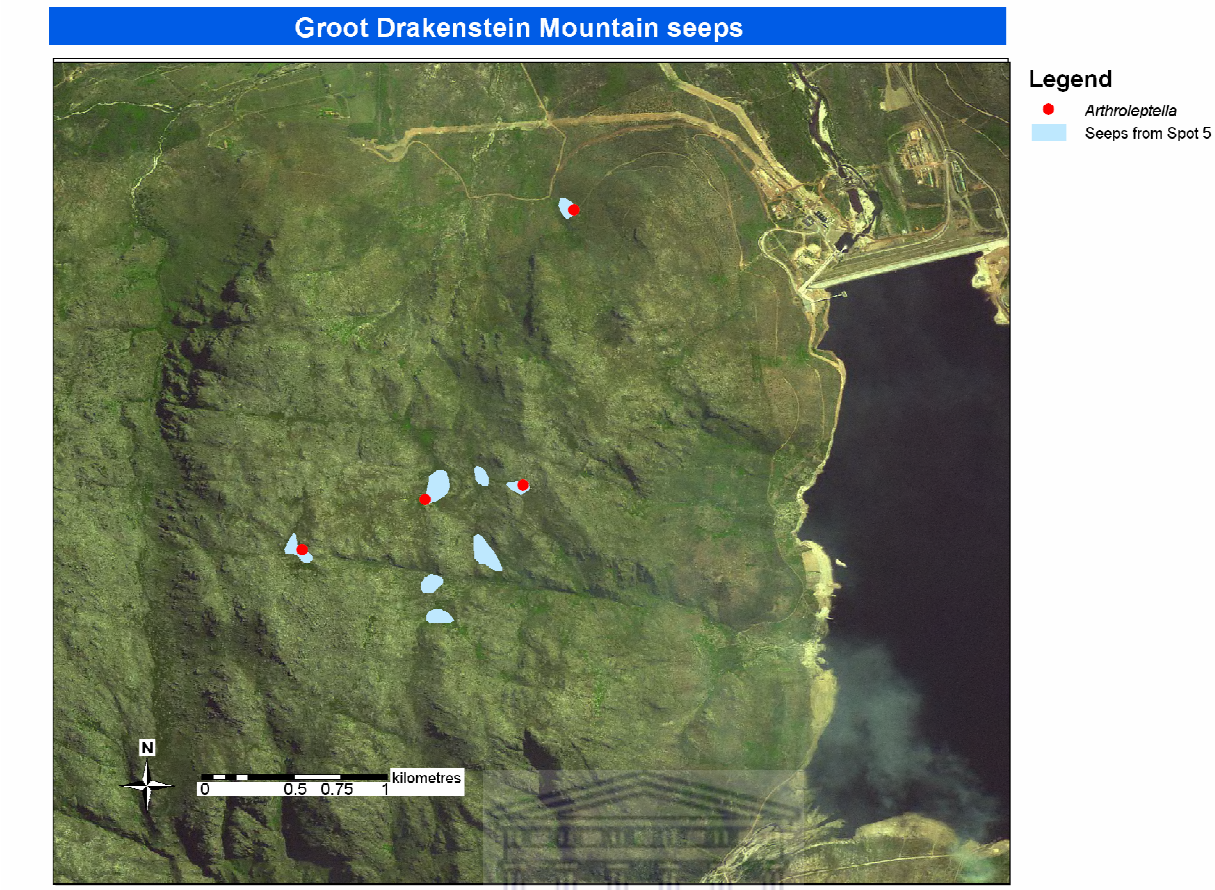

Figure 11. Map showing an example (Groot Drakenstein Mountains) of the way mountain seeps are distributed in the CFM with known *Arthroleptella* populations overlain.

A count of the number of frog species in the western (winter rainfall) versus the eastern (all year rain, see Figure 4) CFR totalled 32 species in the west versus 21 species in the east. Of the frogs endemic to the CFR, 25 are found in the western CFR and eight in the eastern CFR (Table 4).

Table 4. Frog species known to occur in the western and eastern CFR (from Minter *et al.* 2004; Du Preez & Carruthers 2009; own observations). Frogs endemic to the CFR are marked with an asterisk.

Western CFR	Eastern CFR
Amietia fuscigula	*Afrixalus knysnae**
*Amietophrynus pantherinus**	*Amietia angolensis*
Amietophrynus rangeri	*Amietia fuscigula*
*Arthroleptella bicolor**	*Amietia vandijki**
*Arthroleptella drewesii**	*Amietophrynus pardalis*
*Arthroleptella landdrosia**	*Amietophrynus rangeri*
*Arthroleptella lightfooti**	*Breviceps fuscus**
*Arthroleptella subvoce**	*Breviceps montanus**
*Arthroleptella villiersi**	*Cacosternum boettgeri*
*Breviceps acutirostris**	*Cacosternum nanum*
*Breviceps gibbosus**	*Capensibufo tradouwi**
*Breviceps montanus**	*Heleophryne orientalis**
*Breviceps rosei**	*Heleophryne regis**
*Cacosternum capense**	*Hyperolius horstocki*
*Cacosternum platys**	*Hyperolius marmoratus*
*Capensibufo rosei**	*Semnodactylus wealii*
*Capensibufo tradouwi**	*Strongylopus fasciatus*
*Heleophryne depressa**	*Strongylopus grayii*
*Heleophryne purcelli**	*Tomopterna delalandii*
*Heleophryne rosei**	*Vandijkophrynus angusticeps**
*Hyperolius horstocki**	*Xenopus laevis*
*Microbatrachella capensis**	
*Poyntonia paludicola**	
Semnodactylus wealii	
*Strongylopus bonaespei**	
Strongylopus fasciatus	
Strongylopus grayii	
Tomopterna delalandii	
*Vandijkophrynus angusticeps**	
*Xenopus gilli**	
Xenopus laevis	

3.2 Calls

In this section I describe the calls from all of the *Arthroleptella* populations sampled and compare them to the calls from the different populations. The influences of geographic location, as

represented by the mountain range from which calls were recorded, and phylogenetic clade on call characters and variation are examined. In addition, the variation between populations and within individual males is described. A total of 240 individual male frogs were recorded yielding a total of 5 842 advertisement calls (see Table 5 for sample sizes per mountain range).

Table 5. Sample sizes for call measurements for each taxon grouped per mountain range.

Species	Mountain range	Individual n	Call n
Arthroleptella sp. A. West	Riviersonderendberg	17	495
Arthroleptella sp. A East	Riviersonderendberg	16	380
Arthroleptella bicolor	Limietberg	20	424
Arthroleptella drewesii	Babilonstoring	10	266
Arthroleptella drewesii	Kleinriviersberg	5	106
*Arthroleptella landdrosia "*Houwhoek*"*	Groenlandberg	5	149
*Arthroleptella landdrosia "*Houwhoek*"*	Houwhoek	10	280
Arthroleptella sp. B	Du Toitskloof	20	483
Arthroleptella sp. B	Klein Drakenstein	10	271
Arthroleptella sp. C	Kogelberg	15	365
Arthroleptella landdrosia	Hottentots-Holland	22	398
Arthroleptella landdrosia	Simonsberg	6	157
Arthroleptella lightfooti	Cape Point	10	294
Arthroleptella lightfooti	Constantiaberg	11	322
Arthroleptella rugosa	Caledon Swartberg	16	399
Arthroleptella subvoce	Grootwinterhoek	26	423
Arthroleptella villiersi	Hermanus	1	30
Arthroleptella villiersi	Babilonstoring	5	150
Arthroleptella villiersi	Groot Drakenstein	6	180
Arthroleptella villiersi	Hottentots-Holland	9	270

3.2.1 Temperature effects

Temperature is well known to affect calls made by frogs as poikilothermic animals. Typically there are positive relationships between temperature and call rate, note rate and dominant frequency and negative relationships between temperature and call duration (Blair 1955; 1958; Littlejohn 1965; Sullivan 1982; Gayou 1984; Minter 1995). The influence of temperature on the call characters of *Arthroleptella* species is represented by the x-y plots with linear regressions fitted in Figures 12 to 19. The effect of temperature on calls of the genus (all populations pooled) is significant for all variables measured and was positive for note rate, notes per call, pulse rate and pulses per call. It was negative for dominant frequency, call duration and pulses per note. However, all the relationships are weak (see R^2 values in Figures 12 to 19).

Call duration showed negative relationships with temperature for all species. All species showed significant relationships except *A. landdrosia* and *A. rugosa*.

Dominant frequency showed negative relationships with temperature for all species. All species showed significant relationships except *A. sp.* A and *A. sp.* B. This result may be expected due to the close relationship between these two taxa.

Note rate showed positive relationships with temperature for all species. All species showed significant relationships except *A. subvoce.*

Notes per call showed positive relationships with temperature for all species. All species showed significant relationships except all members of the Chirping clade (*A. lightfooti, A. rugosa* and *A. villiersi*). This result is expected for *A. lightfooti* and *A. villiersi* as they only have one note per call.

Pulse rate showed positive relationships with temperature for all species. All species showed significant relationships except *A. landdrosia* and *A. rugosa*.

Pulses per call showed positive relationships with temperature for all species. All species showed significant relationships except *A. sp.* A West, *A. lightfooti, A. rugosa* and *A. villiersi*.

Pulses per note showed negative relationships with temperature for all species. All species showed significant relationships except *A. sp.* A, *A. bicolor, A. sp.* B, *A. sp.* C and *A. rugosa.*

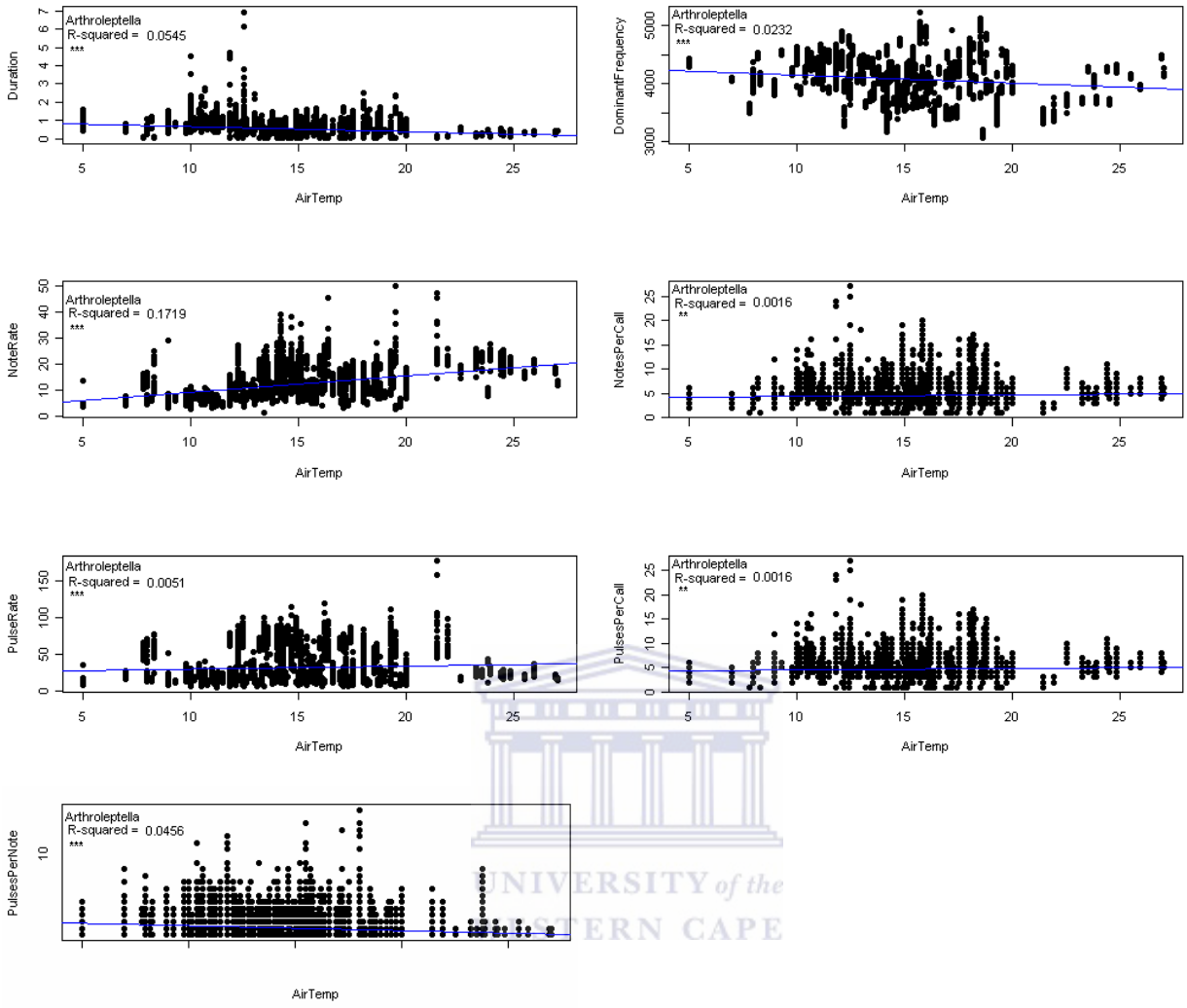

Figure 12. Linear regressions of call features on temperature for the genus *Arthroleptella*. Significance of fitted line denoted as follows: p-values <0.001 ***, <0.01 ** and <0.05 *.

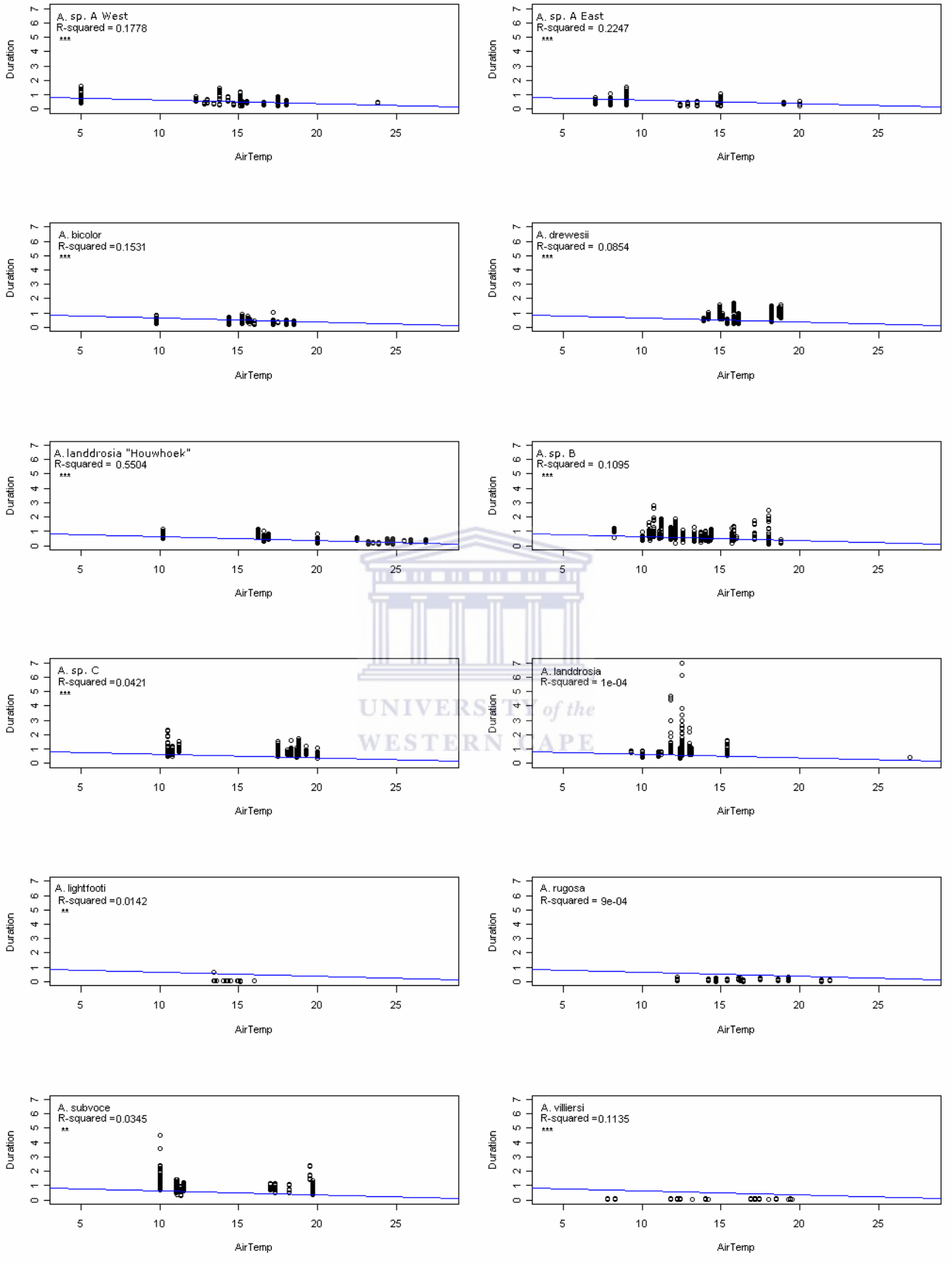

Figure 13. Linear regressions of call duration on temperature for individual *Arthroleptella* species.

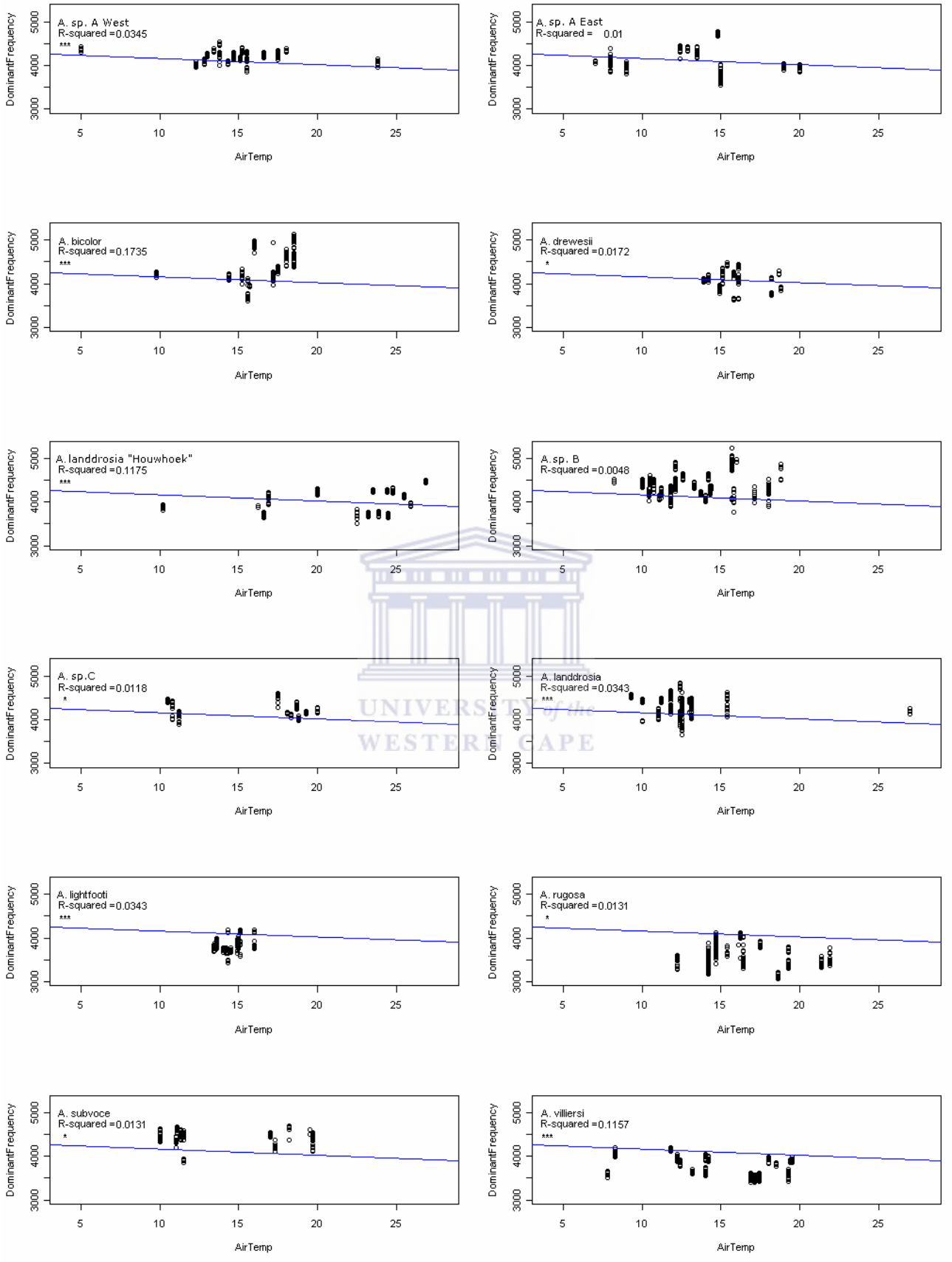

Figure 14. Linear regressions of call dominant frequency on temperature for individual *Arthroleptella* species.

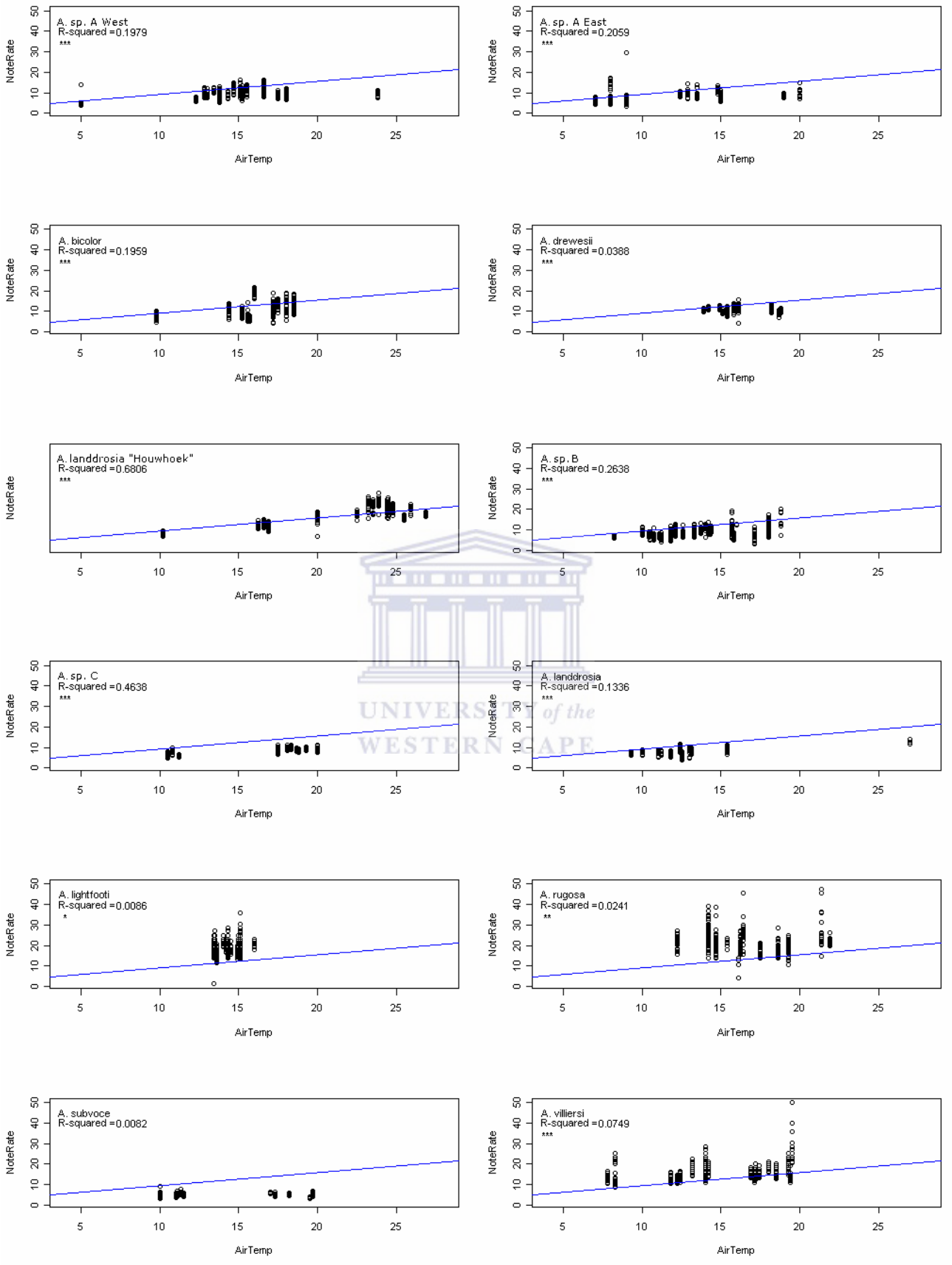

Figure 15. Linear regressions of call note rate on temperature for individual *Arthroleptella* species.

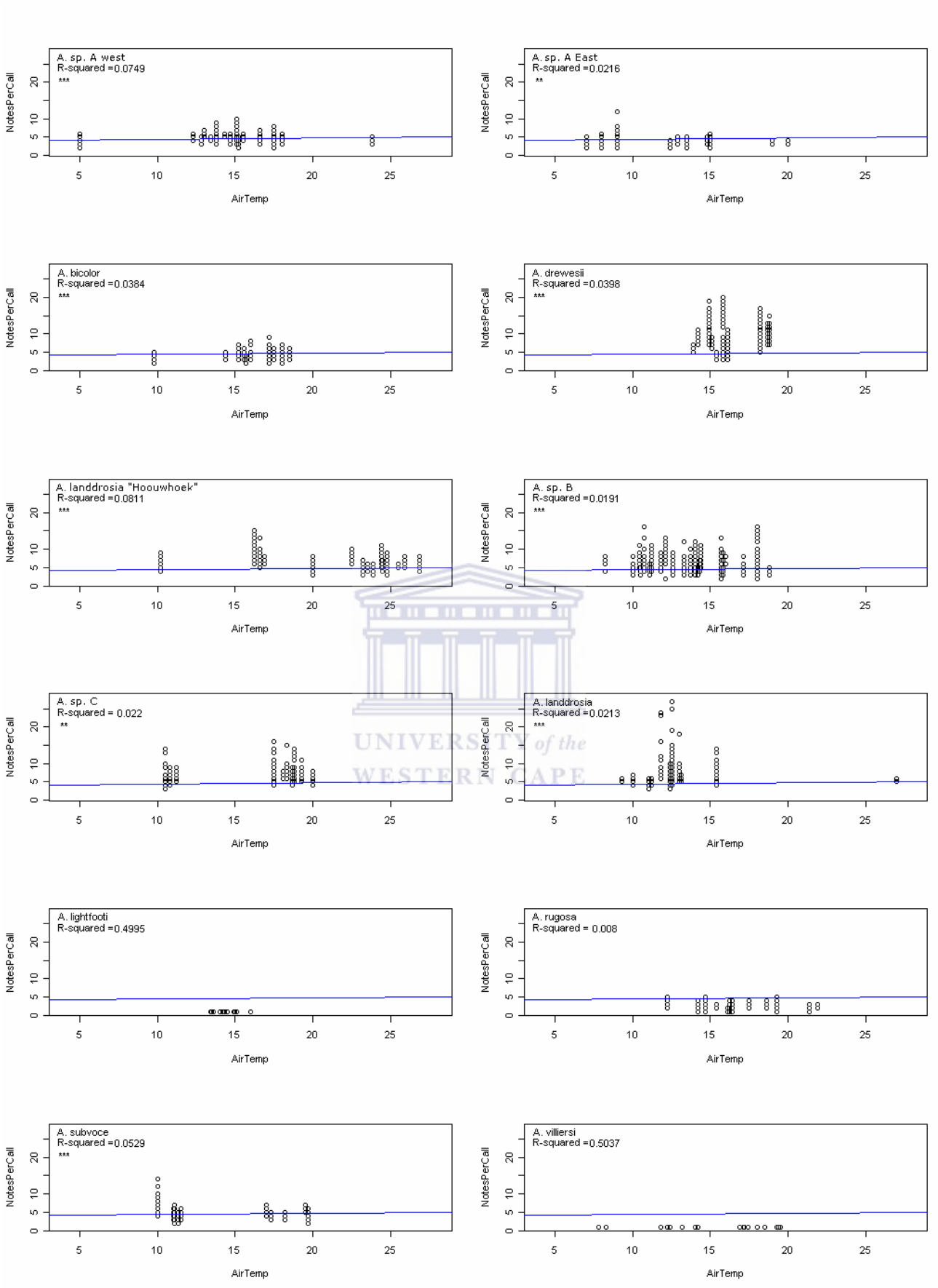

Figure 16. Linear regressions of call notes per call on temperature for individual *Arthroleptella* species.

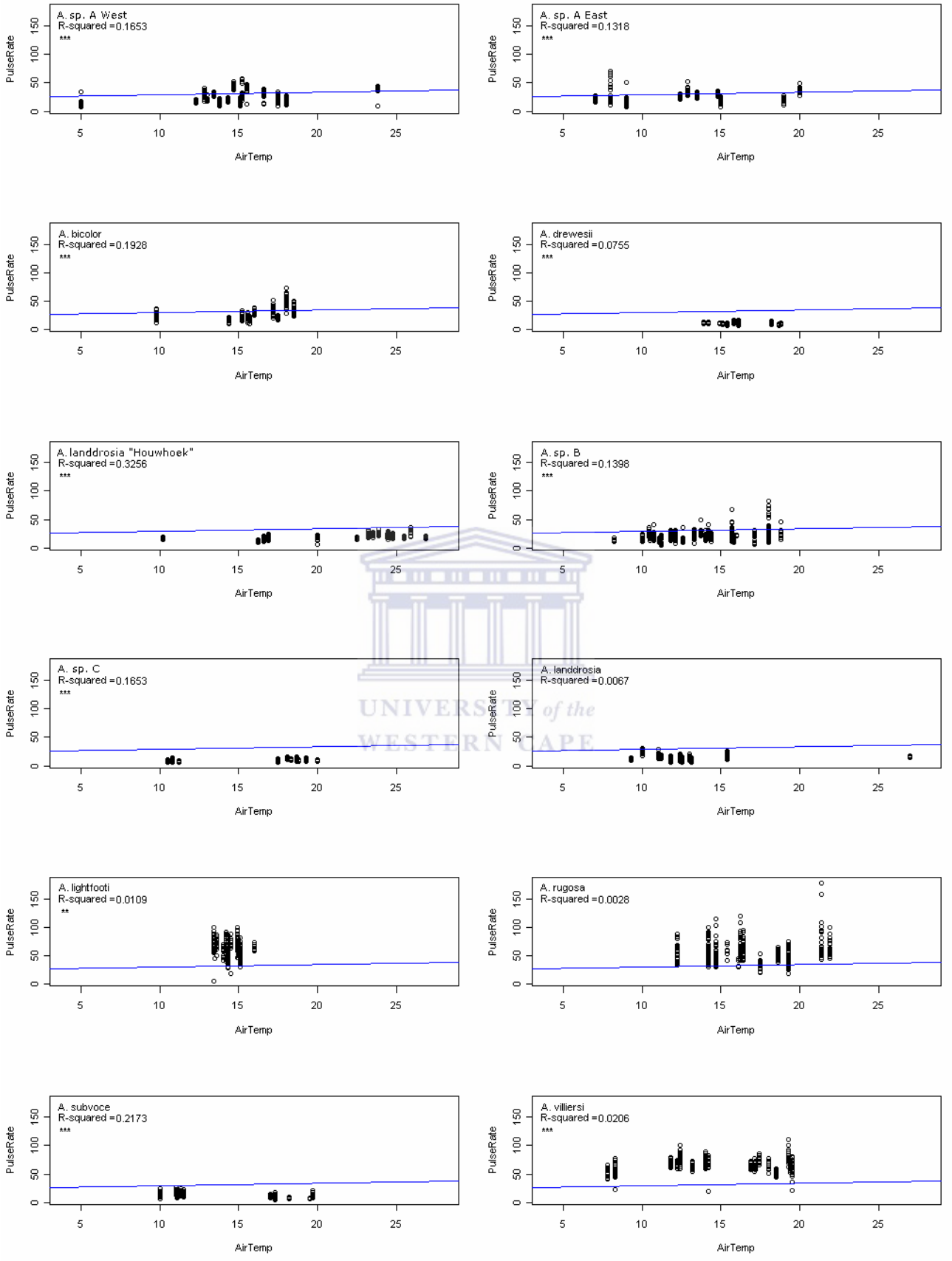

Figure 17. Linear regressions of call pulse rate on temperature for individual *Arthroleptella* species.

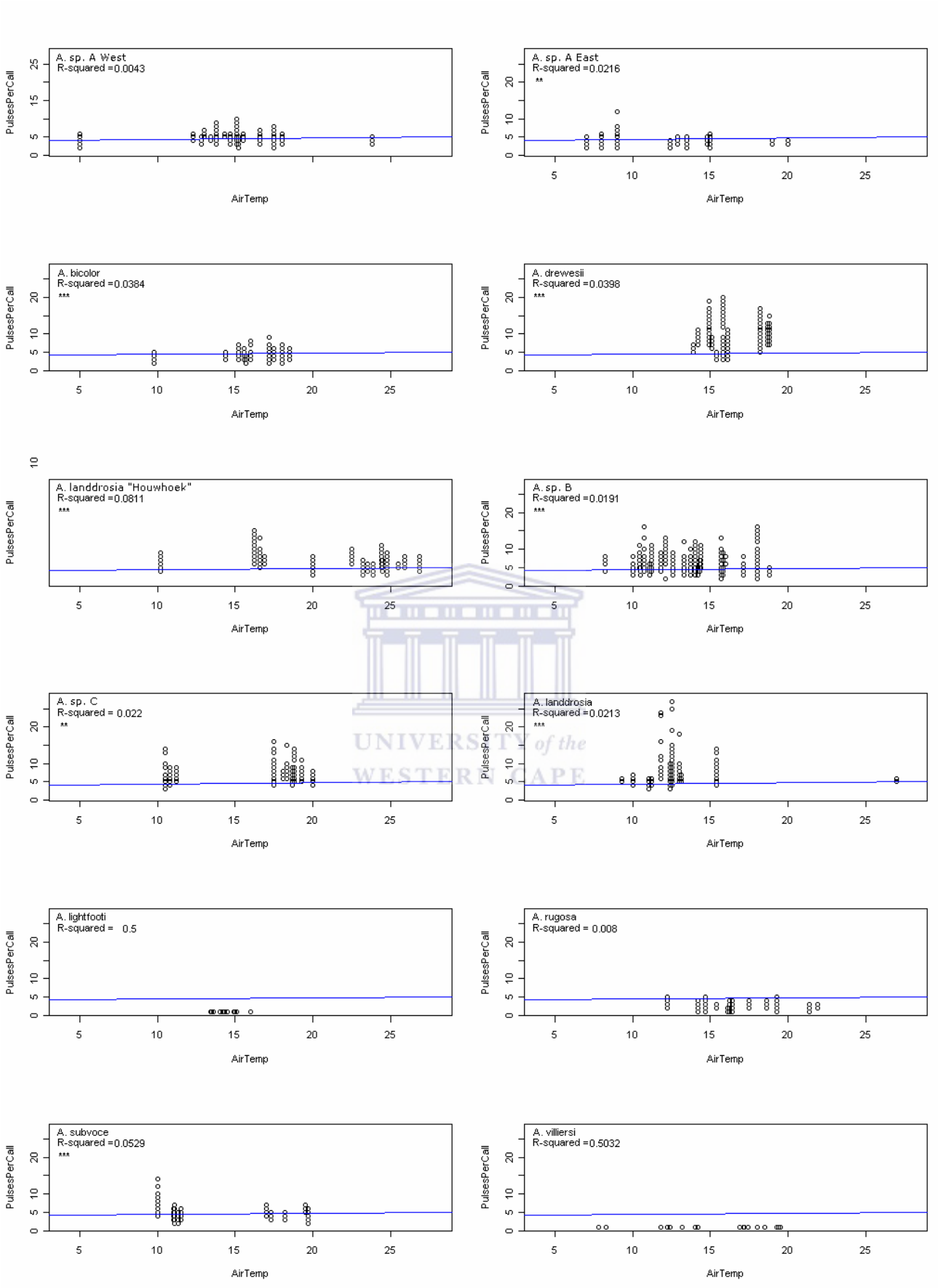

Figure 18. Linear regressions of call pulses per call on temperature for individual *Arthroleptella* species.

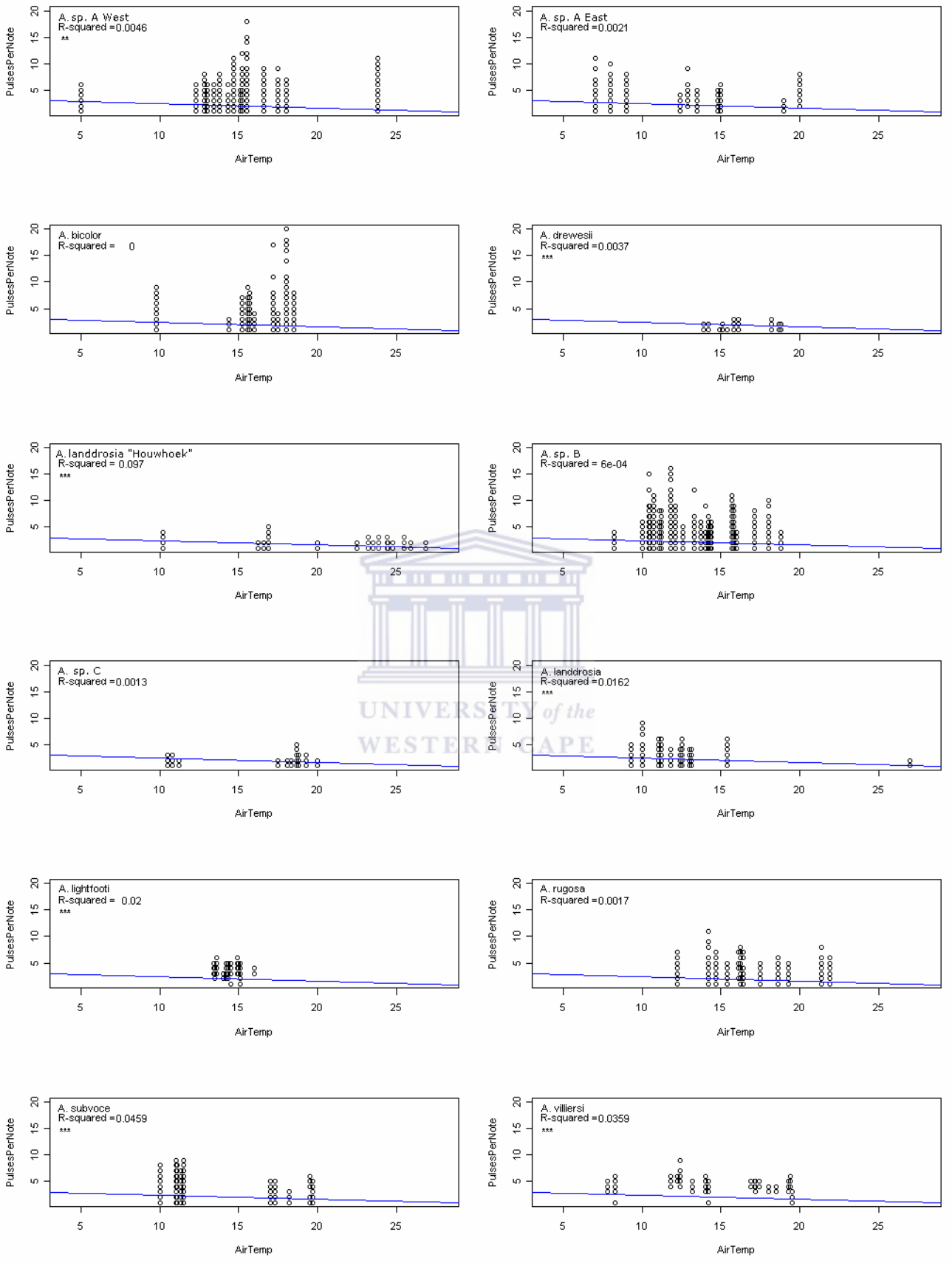

Figure 19. Linear regressions of call pulses per note on temperature for individual *Arthroleptella* species.

The strength of the relationships between each call variable and temperature as judged by R-squared values was weak for all species (see Figures 12 to 19). The variation in the measured variables for a given temperature indicates that factors other than temperature are involved. It also means that it is not yet possible to construct predictive models of these variables based on temperature as the R-squared values are too low to extrapolate using the fitted regression equations with confidence. This is due to the increasing error as extrapolation moves away from known points (Boecklen & Gotelli 1984). Thus although temperature does have an effect on call measurements it is not possible to remove this effect from call variable comparisons. As little variation in the call measurements can be explained by temperature, this lack is not considered to be of serious consequence to the current investigation.

3.2.2 Call measurements

Dominant frequency

The advertisement calls of *A. rugosa* have significantly lower average dominant frequency than all other *Arthroleptella* species (Table 6). However, a single population of *A. villiersi* from Babilonstoring has an even lower average dominant frequency (Table 7).

A. lightfooti and *A. villiersi* have very similar average dominant frequencies but there is marked variation in this trait between populations (compare mountain ranges within *A. villiersi* in Table 7 and notice significant differences across mountain ranges in Table 22).

A. landdrosia "Houwhoek", *A. drewesii* and *A. sp.* A all have similar dominant frequencies (Table 7) but only *A. landdrosia* "Houwhoek" and *A. drewesii* do not differ significantly (Table 23 and Table 24). *Arthroleptella sp.* C and *A. sp.* A West also have similar dominant frequencies (Table 7) and the differences are not statistically significant (Table 23). *Arthroleptella landdrosia*, *A. bicolor* and *A. sp.* B all have similar dominant frequencies (Table 7) with only *A. bicolor* and *A. sp.* B showing a significant difference (Table 23). *Arthroleptella subvoce* has a significantly higher dominant frequency than all other species except *A. landdrosia* (see Table 7 and Table 23). This result may be expected from the smaller body size of this species as small body size is associated with higher frequency (Zweifel 1968; Ryan 1980; Doherty & Gerhardt 1984).

Table 6. Dominant frequency of advertisement calls (mean, standard deviation) for each *Arthroleptella* species. The Houwhoek population of *A. landdrosia* is examined separately.

Species	Mean dominant frequency (Hz)	Std. dev. dominant frequency (Hz)
Arthroleptella sp. A West	4222.46	141.00
Arthroleptella sp. A East	4098.03	273.33
Arthroleptella bicolor	4323.87	338.48
Arthroleptella drewesii	4048.50	245.97
Arthroleptella landdrosia "Houwhoek"	4018.19	244.27
Arthroleptella sp. B	4334.57	231.73
Arthroleptella sp. C	4212.25	186.78
Arthroleptella landdrosia	4304.94	234.80
Arthroleptella lightfooti	3794.40	173.03
Arthroleptella rugosa	3577.30	267.66
Arthroleptella subvoce	4452.15	154.75
Arthroleptella villiersi	3801.52	213.24

Table 7. Dominant frequency of advertisement calls (mean, standard deviation) for each *Arthroleptella* species from each mountain range sampled.

Species	Mountain range	Mean dominant frequency (Hz)	Std. dev. dominant frequency (Hz)
A. sp. A West	Riviersonderendberg	4222.46	141.00
A. sp. A East	Riviersonderendberg	4098.03	273.33
A. bicolor	Limietberg	4323.87	338.48
A. drewesii	Babilonstoring	3978.12	245.37
A. drewesii	Kleinriviersberg	4225.10	133.39
A. landdrosia "Houwhoek"	Groenlandberg	3916.46	149.09
*A. landdrosia "*Houwhoek*"*	Houwhoek	4072.33	266.97
A. sp. B	Du Toitskloof	4320.61	214.23
A. sp. B	Klein Drakenstein	4359.42	258.52
A. sp. C	Kogelberg	4212.25	186.78
A. landdrosia	Hottentots-Holland	4316.00	227.44
A. landdrosia	Simonsberg	4276.89	251.09
A. lightfooti	Cape Point	3838.04	83.97
A. lightfooti	Constantiaberg	3754.30	218.37
A. rugosa	Caledon Swartberg	3577.30	267.66
A. subvoce	Grootwinterhoek	4452.15	154.75
A. villiersi	Hermanus	3666.43	35.79
A. villiersi	Babilonstoring	3528.81	71.61
A. villiersi	Groot Drakenstein	3835.84	147.85
A. villiersi	Hottentots-Holland	3930.15	158.13

Call Duration

All species have significantly different call durations from all other species with the exception of *A. drewesii* and *A. sp.* C*; A. bicolor* and *A. landdrosia* "Houwhoek"; and the parapatric species pair *A. sp.* A. West and *A. sp.* A East (Tables 25, 26 and 27). *Arthroleptella lightfooti* has the shortest calls on average closely followed by *A. villiersi. Arthroleptella rugosa* also has short calls but they are much longer than either *A. lightfooti* or *A. villiersi* (Table 9). Call durations get progressively longer through *A. sp.* B, *A. drewesii*, *A. sp.* C and *A. landdrosia* respectively. *Arthroleptella subvoce* has longer average call durations than all other species (Table 9).

Table 8. Advertisement call duration (mean, standard deviation) for each *Arthroleptella* species.

Species	Mean duration (s)	Std. dev. duration (s)
Arthroleptella sp. A West	0.539	0.225
Arthroleptella sp. A East	0.522	0.220
Arthroleptella bicolor	0.420	0.146
Arthroleptella drewesii	0.808	0.330
Arthroleptella landdrosia "Houwhoek"	0.415	0.208
Arthroleptella sp. B	0.732	0.340
Arthroleptella sp. C	0.844	0.348
Arthroleptella landdrosia	0.966	0.646
Arthroleptella lightfooti	0.058	0.045
Arthroleptella rugosa	0.121	0.049
Arthroleptella subvoce	0.981	0.387
Arthroleptella villiersi	0.063	0.016

Table 9. Advertisement call duration (mean, standard deviation) for each *Arthroleptella* species from each mountain range sampled.

Species	Mountain range	Mean duration (s)	Std. dev. duration (s)
Arthroleptella sp. A West	Riviersonderendberg	0.52	0.22
Arthroleptella sp. A East	Riviersonderendberg	0.54	0.22
Arthroleptella bicolor	Limietberg	0.42	0.15
Arthroleptella drewesii	Babilonstoring	0.87	0.33
Arthroleptella drewesii	Kleinriviersberg	0.60	0.19
Arthroleptella landdrosia "Houwhoek"	Groenlandberg	0.67	0.18
Arthroleptella landdrosia "Houwhoek"	Houwhoek	0.31	0.10
Arthroleptella sp. B	Du Toitskloof	0.80	0.38
Arthroleptella sp. B	Klein Drakenstein	0.60	0.20
Arthroleptella sp. C	Kogelberg	0.84	0.35
Arthroleptella landdrosia	Hottentots-Holland	0.97	0.66
Arthroleptella landdrosia	Simonsberg	0.72	0.21
Arthroleptella lightfooti	Cape Point	0.06	0.06
Arthroleptella lightfooti	Constantiaberg	0.05	0.01
Arthroleptella rugosa	Caledon Swartberg	0.12	0.05
Arthroleptella subvoce	Grootwinterhoek	0.98	0.39
Arthroleptella villiersi	Hermanus	0.06	0.00
Arthroleptella villiersi	Babilonstoring	0.06	0.01
Arthroleptella villiersi	Groot Drakenstein	0.06	0.01
Arthroleptella villiersi	Hottentots-Holland	0.06	0.02

Note rate

Arthroleptella subvoce has the slowest note rate of all *Arthroleptella* species (Table 10 and Table 11). Next slowest is *A. landdrosia* followed by *A. sp.* A. East, *A. sp.* C and *A. sp.* B. *Arthroleptella sp.* A West has a note rate of just less than 10 notes per second. *Arthroleptella bicolor* and *A. drewesii* have similar note rates at around 11 notes per second. *Arthroleptella villiersi* and *A. landdrosia* "Houwhoek" have very similar note rates. *Arthroleptella villiersi* has a note rate of approximately 19 and *A. rugosa* has the highest note rate of all *Arthroleptella* at over 21 notes per second.

Most species have significantly different note rates with the exception of the following pairwise comparisons: *A. bicolor* and *A. drewesii*; *A. sp.* C and *A. sp.* B; *A. villiersi* and *A. landdrosia* "Houwhoek" (Tables 28, 29 and 30).

Table 10. Advertisement call note rate (mean, standard deviation) for each *Arthroleptella* species.

Species	Mean note rate	Std. dev. note rate
Arthroleptella sp. A West	9.84	2.67
Arthroleptella sp. A East	8.34	2.73
Arthroleptella bicolor	11.24	3.90
Arthroleptella drewesii	11.04	1.31
Arthroleptella landdrosia "Houwhoek"	16.64	4.64
Arthroleptella sp. B	8.84	2.32
Arthroleptella sp. C	8.52	1.48
Arthroleptella landdrosia	7.92	1.59
Arthroleptella lightfooti	18.80	3.33
Arthroleptella rugosa	21.86	5.12
Arthroleptella subvoce	5.12	0.81
Arthroleptella villiersi	16.50	4.24

Table 11. Advertisement call note rate (mean, standard deviation) for each *Arthroleptella* species from each mountain range sampled.

Species	Mountain range	Mean note rate	Std. dev. note rate
Arthroleptella sp. A West	Riviersonderendberg	9.84	2.67
Arthroleptella sp. A East	Riviersonderendberg	8.34	2.73
Arthroleptella bicolor	Limietberg	11.24	3.90
Arthroleptella drewesii	Babilonstoring	11.26	1.27
Arthroleptella drewesii	Kleinriviersberg	10.50	1.26
Arthroleptella landdrosia	Groenlandberg	11.45	2.28
*"*Houwhoek*"*			
Arthroleptella landdrosia	Houwhoek	19.41	2.85
*"*Houwhoek*"*			
Arthroleptella sp. B	Du Toitskloof	8.41	2.43
Arthroleptella sp. B	Klein Drakenstein	9.59	1.90
Arthroleptella sp. C	Kogelberg	8.52	1.48
Arthroleptella landdrosia	Hottentots-Holland	7.43	1.46
Arthroleptella landdrosia	Simonsberg	9.18	1.18
Arthroleptella lightfooti	Cape Point	17.96	3.40
Arthroleptella lightfooti	Constantiaberg	19.58	3.08
Arthroleptella rugosa	Caledon Swartberg	21.86	5.12
Arthroleptella subvoce	Grootwinterhoek	5.12	0.81
Arthroleptella villiersi	Hermanus	16.75	0.96
Arthroleptella villiersi	Babilonstoring	15.83	2.45
Arthroleptella villiersi	Groot Drakenstein	16.12	3.58
Arthroleptella villiersi	Hottentots-Holland	17.11	5.43

Notes per call

Both *A. lightfooti* and *A. villiersi* have single note calls unlike all other *Arthroleptella* (Table 12 and Table 13). The other member of the Chirping clade, *A. rugosa*, averages over two notes per call. All the members of the Clicking clade average more than four notes per call with A. Sp. A East, *A. bicolor*, *A. sp.* A West and *A. subvoce* averaging between 4 and five notes per call, *A. sp.* B, *A. landdrosia* "Houwhoek", *A. landdrosia* and *A. sp.* C average between 6 and 7 notes per call and *A. drewesii* averages over eight notes per call.

Most pairwise species comparisons yielded significant differences. The following pairwise species comparisons were not significantly different: *A. subvoce* and *A. sp.* A East; *A. landdrosia* and *A. landdrosia* "Houwhoek"; *A. landdrosia* and *A. sp.* C; *A. lightfooti* and *A. villiersi*. In the latter three

cases, the similarity of notes per call may well be explained by the close genetic relationships between these species pairs. The lack of a significant difference in the case of *A. subvoce* and *A. sp.* A East cannot be explained by genetic relatedness or geographic proximity.

Table 12. Notes per advertisement call (mean, standard deviation) for each *Arthroleptella* species.

Species	Mean notes per call	Std. dev. notes per call
Arthroleptella sp. A West	4.74	1.20
Arthroleptella sp. A East	4.13	1.21
Arthroleptella bicolor	4.36	1.25
Arthroleptella drewesii	8.71	3.59
Arthroleptella sp. B	6.44	2.05
Arthroleptella sp. C	6.04	2.12
Arthroleptella landdrosia "Houwhoek"	6.94	2.34
Arthroleptella landdrosia	6.67	2.54
Arthroleptella lightfooti	1.00	0.00
Arthroleptella rugosa	2.48	0.74
Arthroleptella subvoce	4.86	1.41
Arthroleptella villiersi	1.00	0.00

Table 13. Notes per advertisement call (mean, standard deviation) for each *Arthroleptella* species from each mountain range sampled.

Species	Mountain range	Mean notes per call	Std. dev. notes per call
Arthroleptella sp. A West	Riviersonderendberg	4.74	1.20
Arthroleptella sp. A East	Riviersonderendberg	4.13	1.21
Arthroleptella bicolor	Limietberg	4.36	1.25
Arthroleptella drewesii	Babilonstoring	9.70	3.60
Arthroleptella drewesii	Kleinriviersberg	6.23	2.02
Arthroleptella landdrosia	Groenlandberg	7.58	2.38
*"*Houwhoek*"*			
Arthroleptella landdrosia	Houwhoek	5.84	1.55
*"*Houwhoek*"*			
Arthroleptella sp. B	Du Toitskloof	6.21	2.18
Arthroleptella sp. B	Klein Drakenstein	5.73	1.99
Arthroleptella sp. C	Kogelberg	6.94	2.34
Arthroleptella landdrosia	Hottentots-Holland	6.72	2.77
Arthroleptella landdrosia	Simonsberg	6.57	1.85
Arthroleptella lightfooti	Cape Point	1.00	0.00
Arthroleptella lightfooti	Constantiaberg	1.00	0.00
Arthroleptella rugosa	Caledon Swartberg	2.48	0.74
Arthroleptella subvoce	Grootwinterhoek	4.86	1.41
Arthroleptella villiersi	Hermanus	1.00	0.00
Arthroleptella villiersi	Babilonstoring	1.00	0.00
Arthroleptella villiersi	Groot Drakenstein	1.00	0.00
Arthroleptella villiersi	Hottentots-Holland	1.00	0.00

Pulse rate

Pulse rates vary considerably (almost sevenfold) across the genus (Table 14 and Table 15). Average pulse rates are distinctive (Table 14) and significantly different for most species with the exception of the pairwise comparisons of *A. sp.* A East and *A. sp.* A West; *A. bicolor* and *A. sp.* A West; *A. lightfooti* and *A. villiersi* which do not show significant differences (Table 35). The average pulse rates of *Arthroleptella lightfooti* and *A. villiersi* are also very close. Pulse rate is lowest in *A. sp.* C and highest in *A. lightfooti* and *A. villiersi*.

Table 14. Advertisement call pulse rate (mean, standard deviation) for each *Arthroleptella* species.

Species	Mean pulse rate	Std. dev. pulse rate
Arthroleptella sp. A West	25.45	10.36
Arthroleptella sp. A East	24.09	10.59
Arthroleptella bicolor	26.66	11.08
Arthroleptella drewesii	11.50	1.63
*Arthroleptella landdrosia "*Houwhoek*"*	19.10	4.90
Arthroleptella sp. B	20.72	8.34
Arthroleptella sp. C	9.75	2.17
Arthroleptella landdrosia	14.19	4.41
Arthroleptella lightfooti	65.66	13.73
Arthroleptella rugosa	52.89	19.19
Arthroleptella subvoce	12.81	3.77
Arthroleptella villiersi	66.37	10.04

Table 15. Advertisement call pulse rate (mean, standard deviation) for each *Arthroleptella* species from each mountain range sampled.

Species	Mountain range	Mean pulse rate	Std. dev. pulse rate
Arthroleptella sp. A West	Riviersonderendberg	25.45	10.36
Arthroleptella sp. A East	Riviersonderendberg	24.09	10.59
Arthroleptella bicolor	Limietberg	26.66	11.08
Arthroleptella drewesii	Babilonstoring	11.54	1.44
Arthroleptella drewesii	Kleinriviersberg	11.39	2.03
Arthroleptella landdrosia	Groenlandberg	14.98	3.24
"Houwhoek"			
Arthroleptella landdrosia	Houwhoek	21.29	4.18
"Houwhoek"			
Arthroleptella sp. B	Du Toitskloof	20.40	9.14
Arthroleptella sp. B	Klein Drakenstein	21.29	6.67
Arthroleptella sp. C	Kogelberg	9.75	2.17
Arthroleptella landdrosia	Hottentots-Holland	13.59	4.80
Arthroleptella landdrosia	Simonsberg	15.72	2.65
Arthroleptella lightfooti	Cape Point	71.13	30.38
Arthroleptella lightfooti	Constantiaberg	60.63	12.60
Arthroleptella rugosa	Caledon Swartberg	52.89	19.19
Arthroleptella subvoce	Grootwinterhoek	12.81	3.77
Arthroleptella villiersi	Hermanus	66.98	3.84
Arthroleptella villiersi	Babilonstoring	69.55	9.06
Arthroleptella villiersi	Groot Drakenstein	71.15	9.05
Arthroleptella villiersi	Hottentots-Holland	61.35	9.37

Pulses per call

Pulses per call are generally distinct (see average values in Tables 16 and 17) and are significantly different across species (Table 38) with the following exceptions. Both *A. landdrosia* and *A. subvoce* do not differ significantly from either of the parapatric species pair *A. sp.* A West and *A. sp.* A East. Also, *A. landdrosia* and *A. subvoce* do not differ significantly from each other.

Table 16. Pulses per advertisement call (mean, standard deviation) for each *Arthroleptella* species.

Species	Mean pulses per call	Std. dev. pulses per call
Arthroleptella sp. A West	12.03	3.70
Arthroleptella sp. A East	11.39	2.94
Arthroleptella bicolor	10.44	3.93
Arthroleptella drewesii	8.99	3.58
Arthroleptella landdrosia "Houwhoek"	7.56	2.67
Arthroleptella sp. B	13.82	5.56
Arthroleptella sp. C	7.92	2.77
Arthroleptella landdrosia	11.79	4.45
Arthroleptella lightfooti	3.59	0.97
Arthroleptella rugosa	5.96	2.27
Arthroleptella subvoce	12.11	4.46
Arthroleptella villiersi	4.22	1.01

Table 17. Pulses per advertisement call (mean, standard deviation) for each *Arthroleptella* species from each mountain range sampled.

Species	Mountain range	Mean pulses per call	Std. dev. pulses per call
Arthroleptella sp. A West	Riviersonderendberg	12.03	3.70
Arthroleptella sp. A East	Riviersonderendberg	11.39	2.94
Arthroleptella bicolor	Limietberg	10.44	3.93
Arthroleptella drewesii	Babilonstoring	9.90	3.62
Arthroleptella drewesii	Kleinriviersberg	6.70	2.17
Arthroleptella landdrosia	Groenlandberg	9.83	2.69
*"*Houwhoek*"*			
Arthroleptella landdrosia	Houwhoek	6.35	1.70
*"*Houwhoek*"*			
Arthroleptella sp. B	Du Toitskloof	14.77	6.32
Arthroleptella sp. B	Klein Drakenstein	12.14	3.26
Arthroleptella sp. C	Kogelberg	7.92	2.77
Arthroleptella landdrosia	Hottentots-Holland	12.07	4.95
Arthroleptella landdrosia	Simonsberg	11.10	2.69
Arthroleptella lightfooti	Cape Point	4.08	0.98
Arthroleptella lightfooti	Constantiaberg	3.15	0.72
Arthroleptella rugosa	Caledon Swartberg	5.96	2.27
Arthroleptella subvoce	Grootwinterhoek	12.11	4.46
Arthroleptella villiersi	Hermanus	4.00	0.00
Arthroleptella villiersi	Babilonstoring	4.44	0.52
Arthroleptella villiersi	Groot Drakenstein	4.61	1.09
Arthroleptella villiersi	Hottentots-Holland	3.87	1.08

Pulses per note

There is less variation in pulses per note than in the other call metrics (Tables 18 and 19). However, there are clear patterns across the species and all species are significantly different from each other except *A. sp.* B and *A. bicolor*; *A. rugosa* and *A. sp.* A West; *A. subvoce* and *A. sp.* A West and *A. subvoce*; *A. sp.* C and *A. landdrosia* "Houwhoek" and *A. rugosa*. Interestingly, none of the pairs of species involved in the non-significant pairwise comparisons are sister taxa.

Table 18. Pulses per note (mean, standard deviation) for each *Arthroleptella* species.

Species	Mean pulses per note	Std. dev. pulses per note
Arthroleptella sp. A West	2.54	1.76
Arthroleptella sp. A East	2.76	1.44
Arthroleptella bicolor	2.39	1.85
Arthroleptella drewesii	1.03	0.19
Arthroleptella landdrosia "Houwhoek"	1.17	0.47
Arthroleptella sp. B	2.29	1.65
Arthroleptella sp. C	1.14	0.38
Arthroleptella landdrosia	1.77	0.89
Arthroleptella lightfooti	3.59	0.97
Arthroleptella rugosa	2.40	1.33
Arthroleptella subvoce	2.49	1.57
Arthroleptella villiersi	4.22	1.01

Table 19. Pulses per note (mean, standard deviation) for each *Arthroleptella* species from each mountain range sampled.

Species	Mountain range	Mean pulses per note	Std. dev. pulses per note
Arthroleptella sp. A West	Riviersonderendberg	2.54	1.76
Arthroleptella sp. A East	Riviersonderendberg	2.76	1.44
Arthroleptella bicolor	Limietberg	2.39	1.85
Arthroleptella drewesii	Babilonstoring	1.02	0.16
Arthroleptella drewesii	Kleinriviersberg	1.08	0.28
Arthroleptella landdrosia	Groenlandberg	1.30	0.62
*"*Houwhoek*"*			
Arthroleptella landdrosia	Houwhoek	1.09	0.30
*"*Houwhoek*"*			
Arthroleptella sp. B	Du Toitskloof	2.38	1.76
Arthroleptella sp. B	Klein Drakenstein	2.12	1.38
Arthroleptella sp. C	Kogelberg	1.14	0.38
Arthroleptella landdrosia	Hottentots-Holland	1.80	0.94
Arthroleptella landdrosia	Simonsberg	1.69	0.75
Arthroleptella lightfooti	Cape Point	4.08	0.98
Arthroleptella lightfooti	Constantiaberg	3.15	0.72
Arthroleptella rugosa	Caledon Swartberg	2.40	1.33
Arthroleptella subvoce	Grootwinterhoek	2.49	1.57
Arthroleptella villiersi	Hermanus	4.00	0.00
Arthroleptella villiersi	Babilonstoring	4.44	0.52
Arthroleptella villiersi	Groot Drakenstein	4.61	1.09
Arthroleptella villiersi	Hottentots-Holland	3.87	1.08

Amplitude modulation

All species show some degree of amplitude modulation. Members of the Clicking clade generally showed increasing or unimodal patterns of amplitude modulation. There was variation within species in this clade so that both increasing and modal patterns were represented.

In the Chirping clade the multi-note calls of *A. rugosa* showed an increasing patter of amplitude modulation. *Arthroleptella lightfooti* and *A. villiersi* produced calls with a decreasing pattern of AM. The general patterns of AM are displayed in Table 20. The variation within populations and species in a categorical factor precludes statistical comparison of this factor of between populations or species and this was not attempted. However, this factor cannot be dismissed as an important call feature and further investigation of amplitude modulation is indicated.

Table 20. Patterns of amplitude modulation over time.

Species	Locality	AM Pattern
A. sp. A West	Amanzi	Increasing/Modal
A. sp. A East	Die Galg	Increasing/Modal
A. sp. A East	Genadendal	Modal
A. sp. A East	Greyton	Increasing/Modal
A. sp. A East	Jonaskop	Modal/Increasing
A. sp. A East	Twistwyk	Increasing/Modal
A. sp. B	Zachariashoek	Increasing/Modal
A. bicolor	Bainskloof	Increasing
A. bicolor	Observation Peak	Increasing/Modal
A. bicolor	Waterval	Increasing/Multimodal/Modal
A. drewesii	All	Modal
A. sp. B	Aasvogelberg	Modal
A. sp. B	Du Toitskloof	Increasing/Modal
A. sp. B	Fizantakraal	Increasing/Modal
A. sp. C	Betty's Bay	Modal
A. sp. C	Koëlberg	Modal/Increasing
A. sp. C	Kogelberg	Modal
A. landdrosia	Groenlandberg	Increasing/Modal
A. landdrosia	Helderberg	Modal
A. landdrosia	Houwhoek	Modal/Increasing
A. landdrosia	Jonkershoek	Modal/Increasing
A. landdrosia	Landdroskop	Modal/Increasing
A. landdrosia	Simonsberg	Modal/Increasing
A. landdrosia	Swartboskloof	Modal
A. landdrosia	Delheim	Modal
A. lightfooti	All	Decreasing
A. rugosa	Caledon	Increasing
A. subvoce	Grootwinterhoek	Increasing/Flat
A. subvoce	Sneeugat	Modal
A. villiersi	All	Decreasing

A. sp. B generally had maximum amplitude in the middle or earlier part of call, and the first few notes may be very quiet. The last few notes may also be relatively quiet in this taxon (Figure 20).

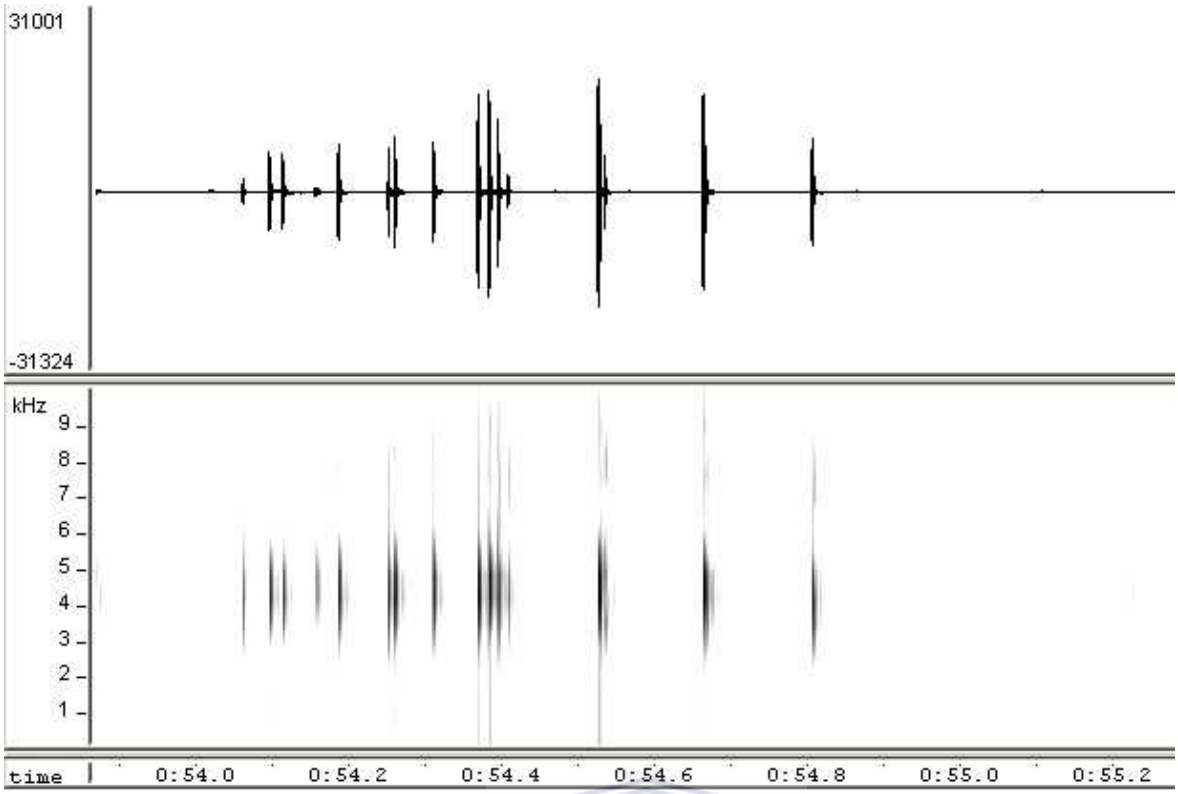

Figure 20. Wave form graph (above) and spectrogram (below) of an *A. sp.* B call showing low amplitude initial and trailing notes.

3.2.3 Spectrograms and waveform diagrams

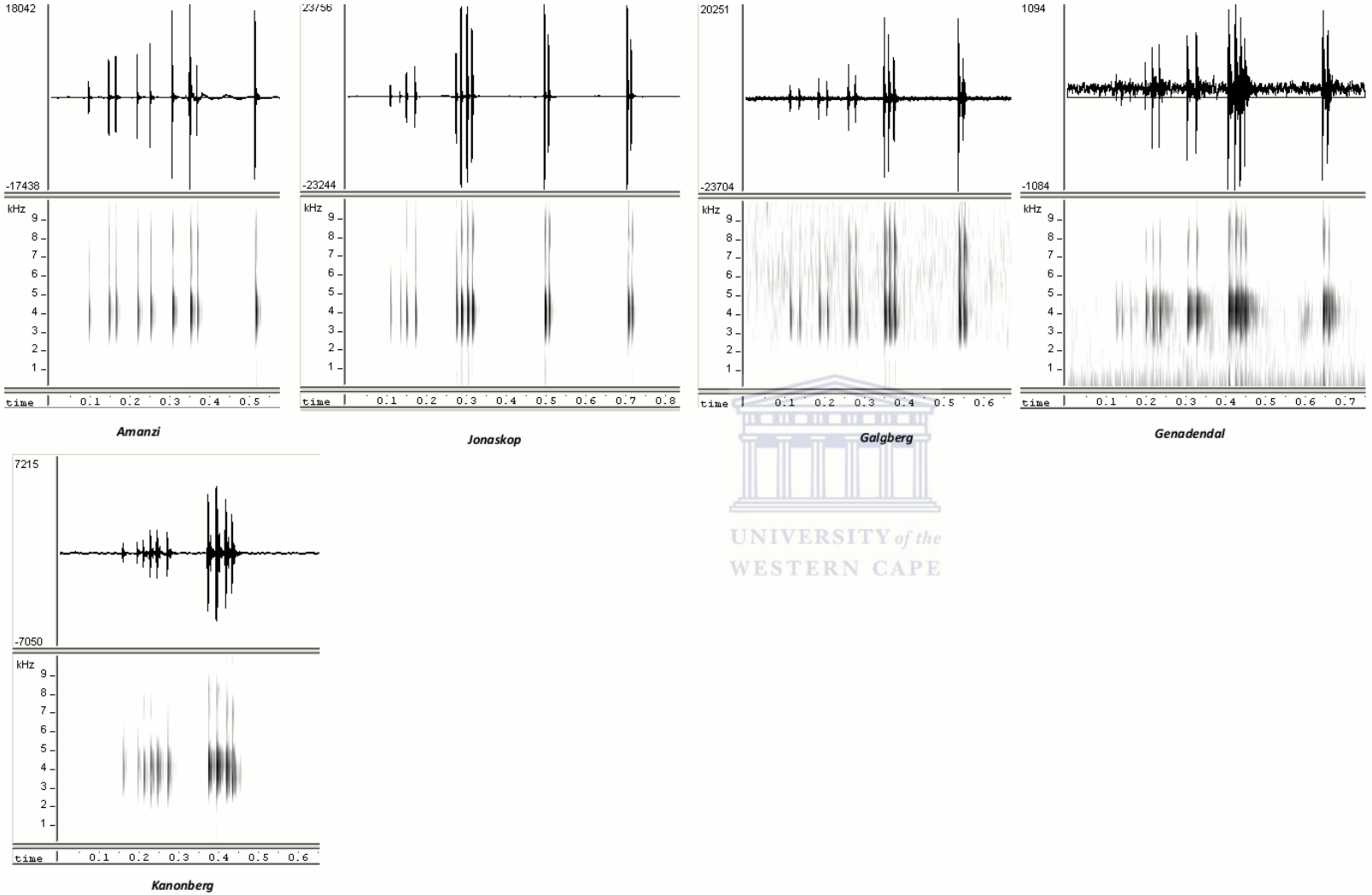

Figure 21. Advertisement calls of *A. sp.* A West from Amanzi and Jonaskop; and *A. sp.* A East from Galgberg, Genadendal and Kanonberg.

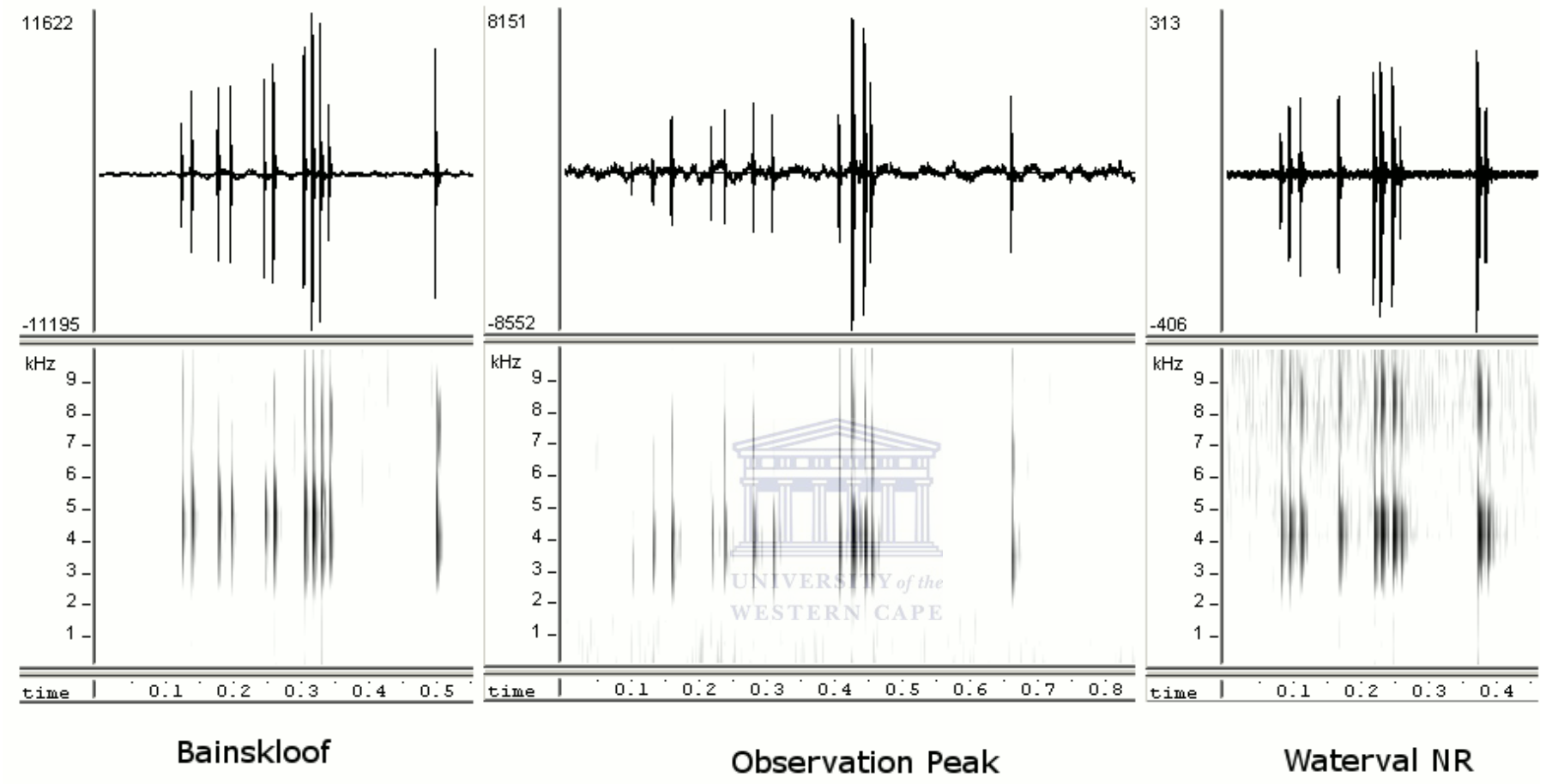

Figure 22. Advertisement calls of *A. bicolor* from Bainskloof, Observation Peak and Waterval Nature Reserve.

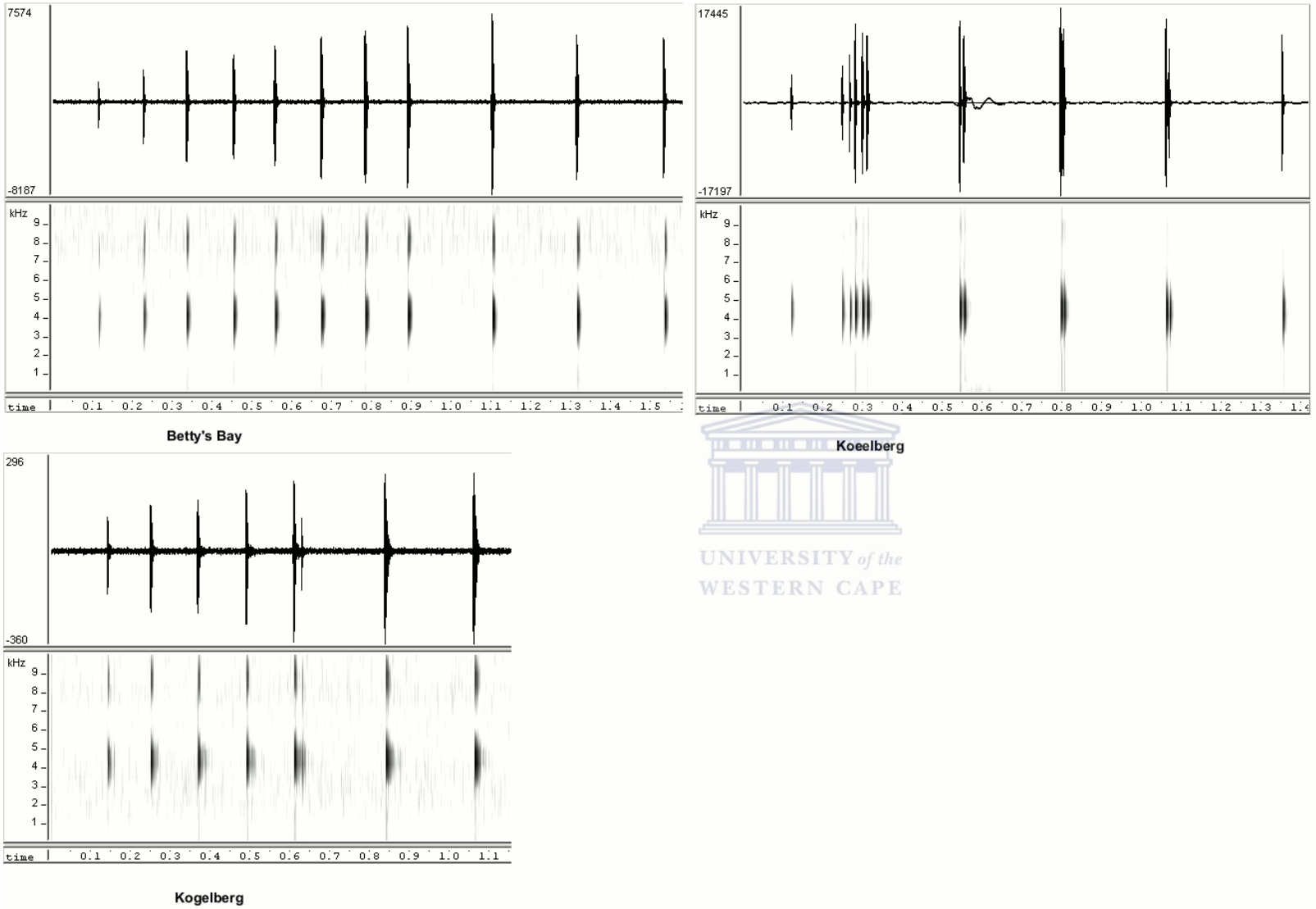

Figure 23. Advertisement calls of *A. sp.* C from Bettty's Bay, Koëlberg and Kogelberg Nature Reserve.

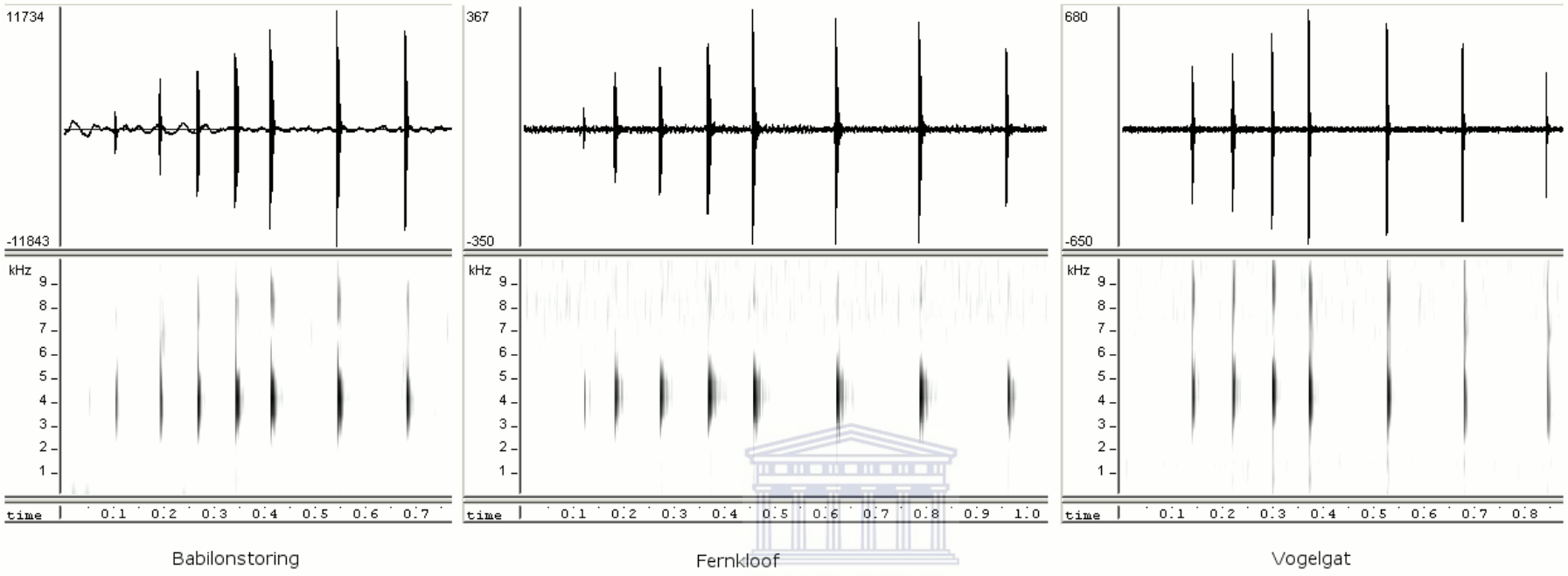

Figure 24. Advertisement calls of *A. drewesii* from Babilonstoring Nature Reserve, Fernkloof Nature Reserve and Vogelgat Private Nature Reserve

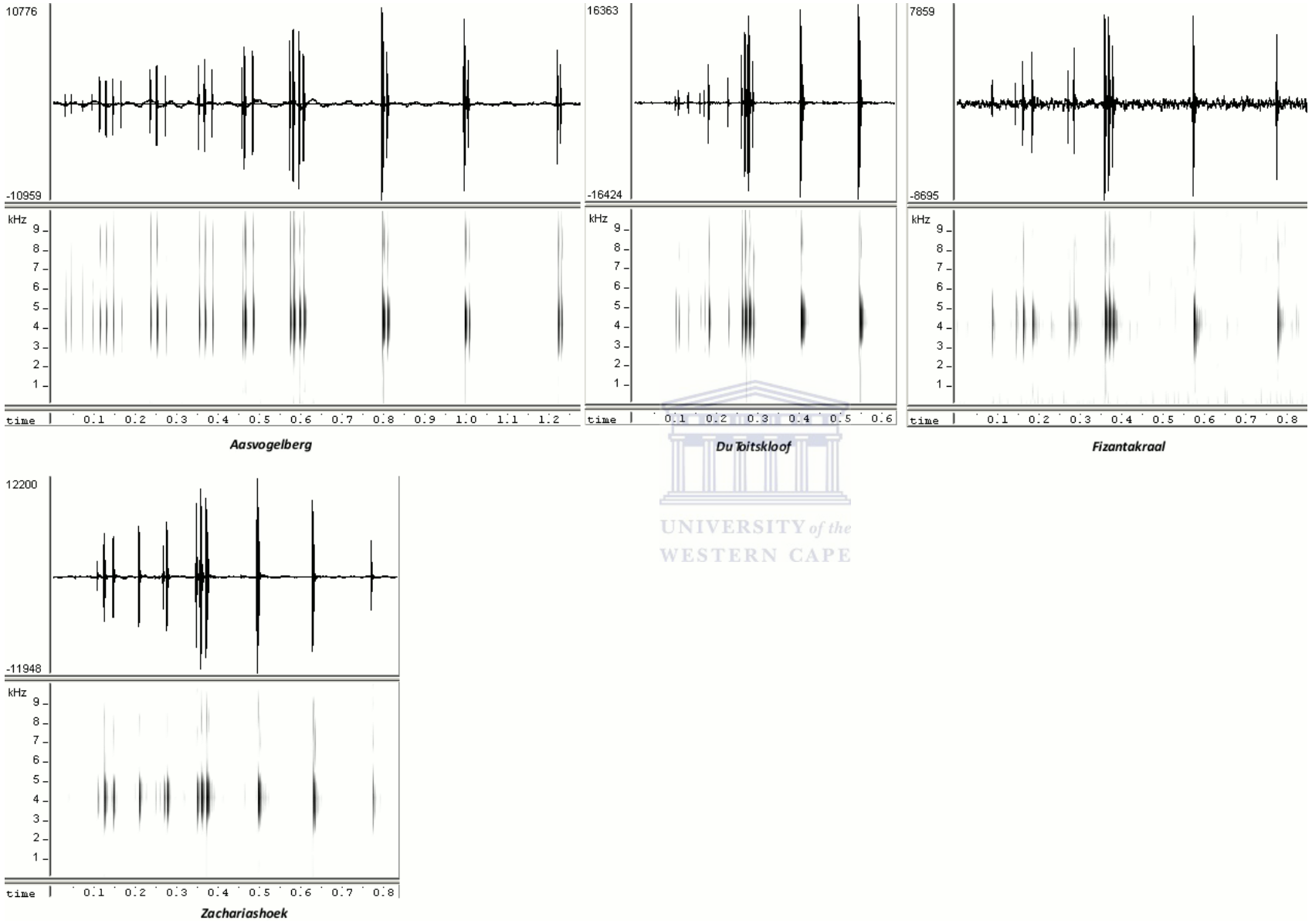

Figure 25. Advertisement calls of *A. sp.* B from Aasvogelberg, Du Toitskloof, Fizantakraal and Zachariashoek.

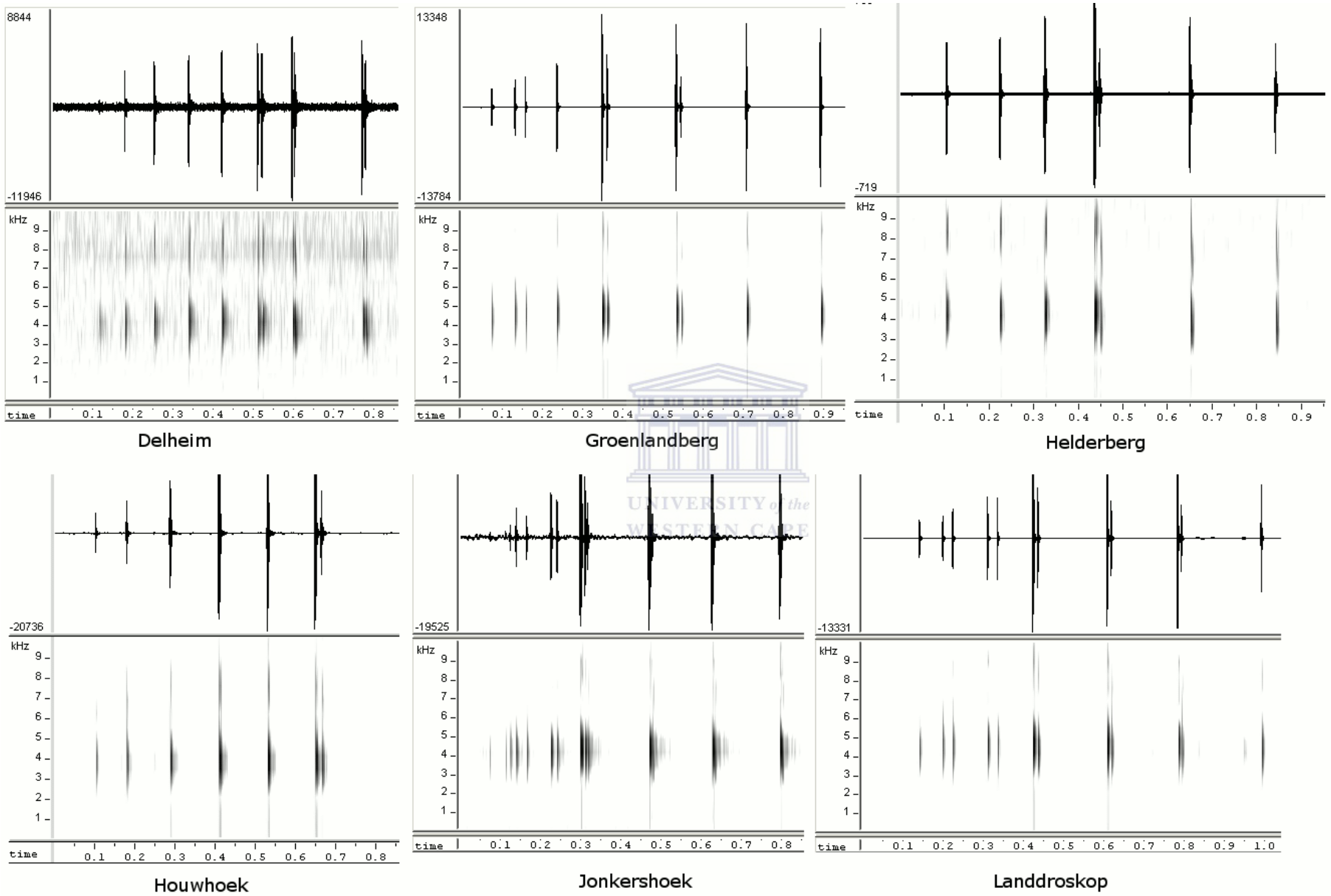

Figure 26. Advertisement calls of *A. landdrosia* from Delheim, Groenlandberg, Helderberg, Houwhoek, Jonkershoek and Landdroskop.

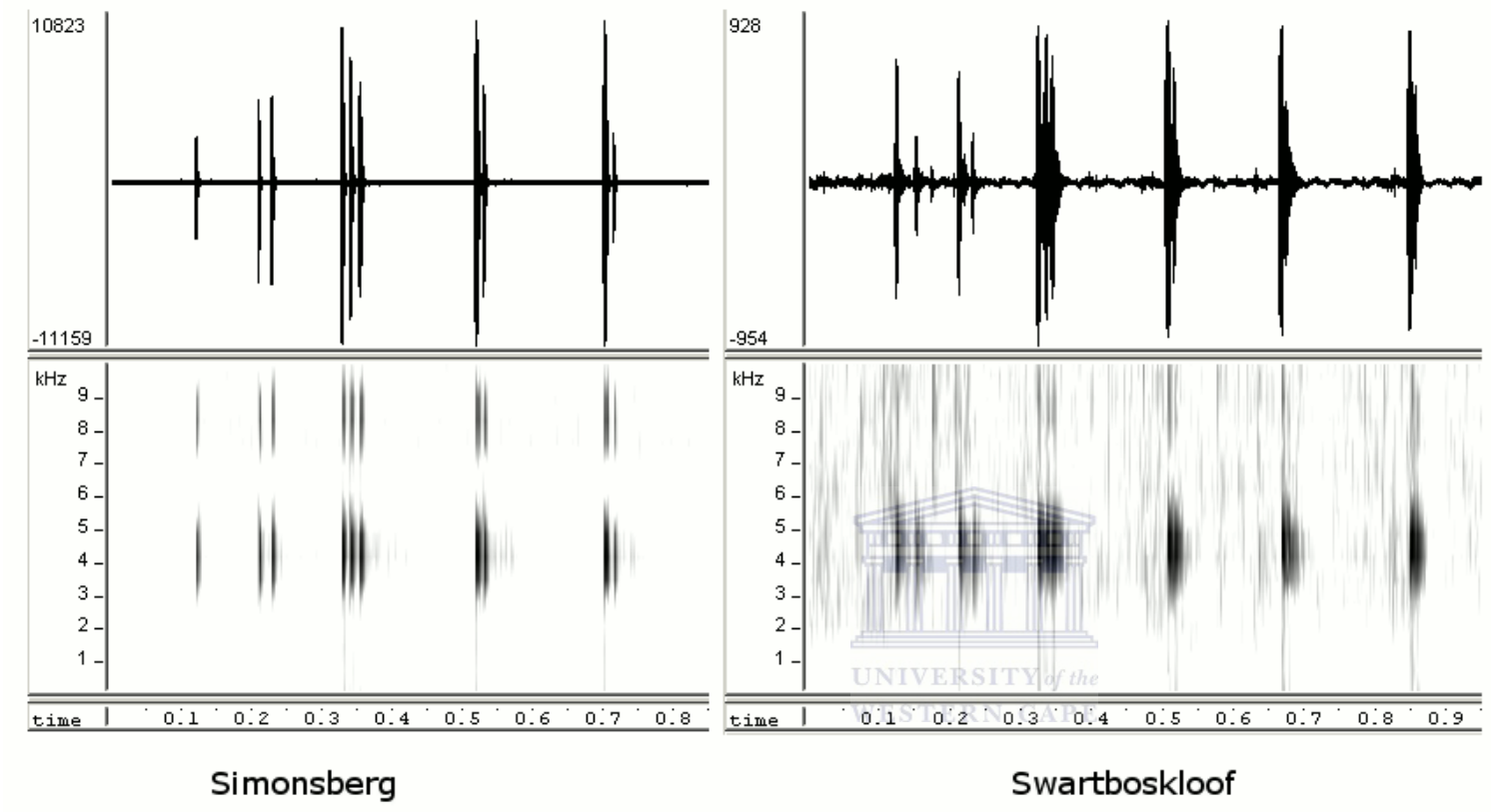

Figure 27. Advertisement calls of *A. landdrosia* from Simonsberg and Swartboskloof.

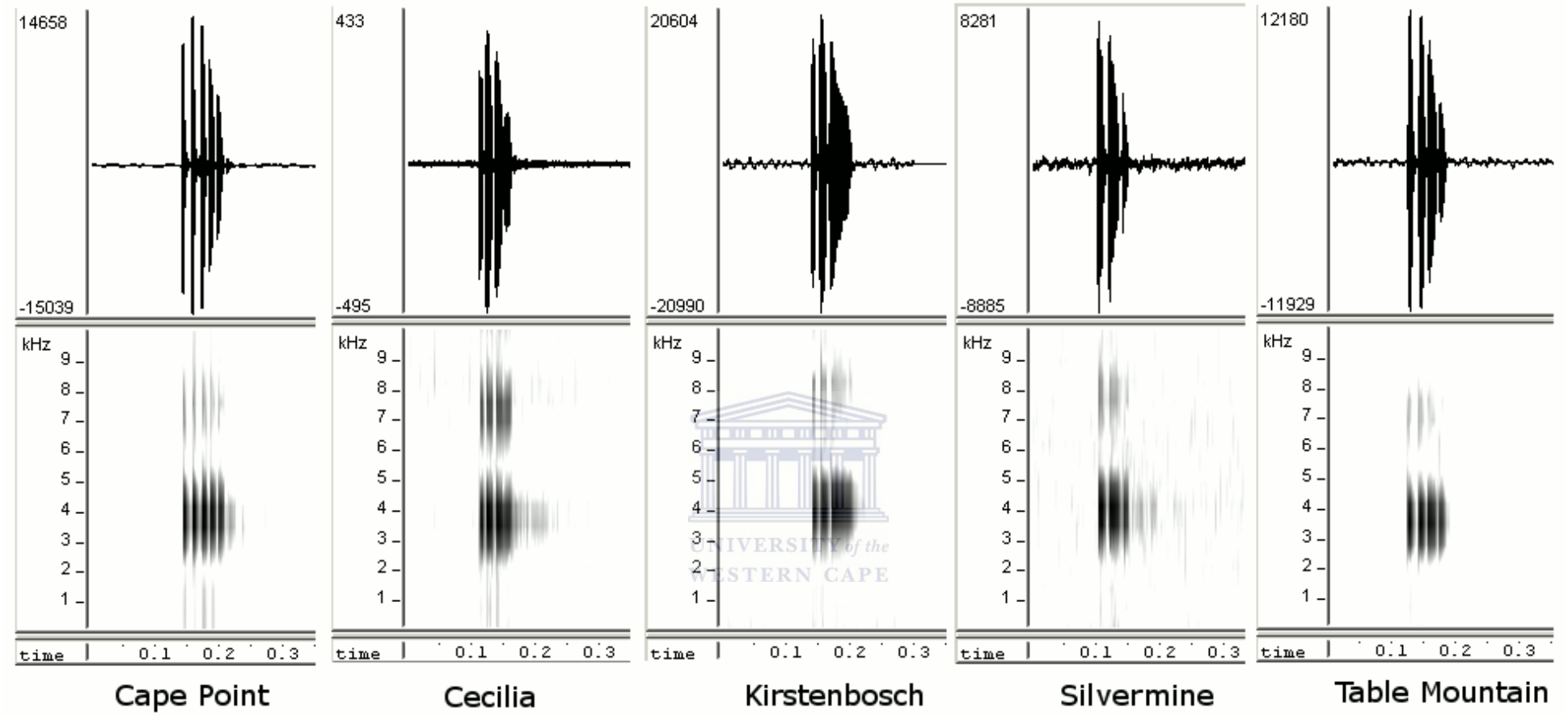

Figure 28. Advertisement calls of *A. lightfooti* from Cape Point, Cecilia plantation, Kirstenbosch, Silvermine and Table Mountain.

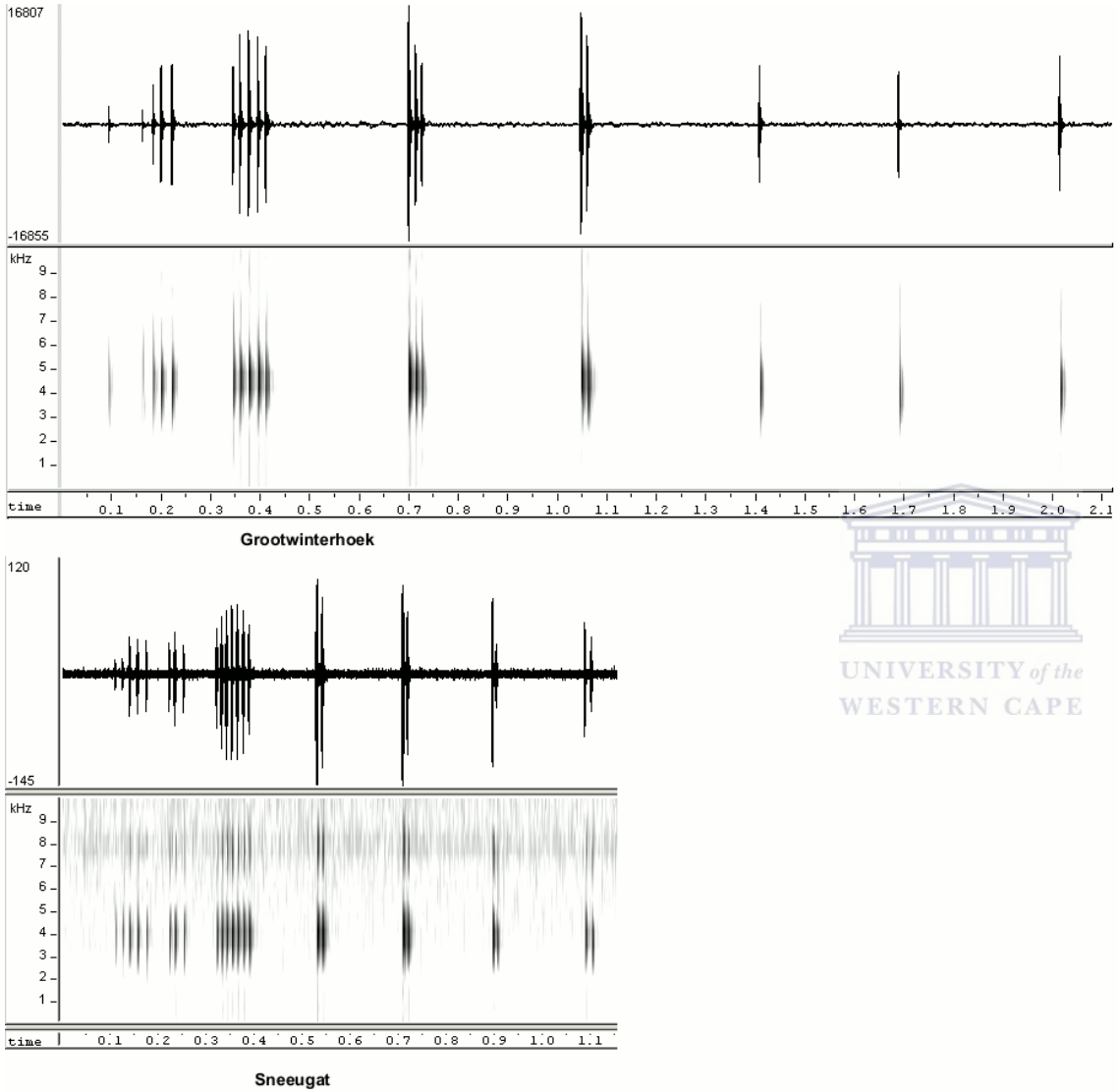

Figure 29. Advertisement calls of *A. subvoce* from Groot Winterhoek Wilderness Area and the Sneeugat trail.

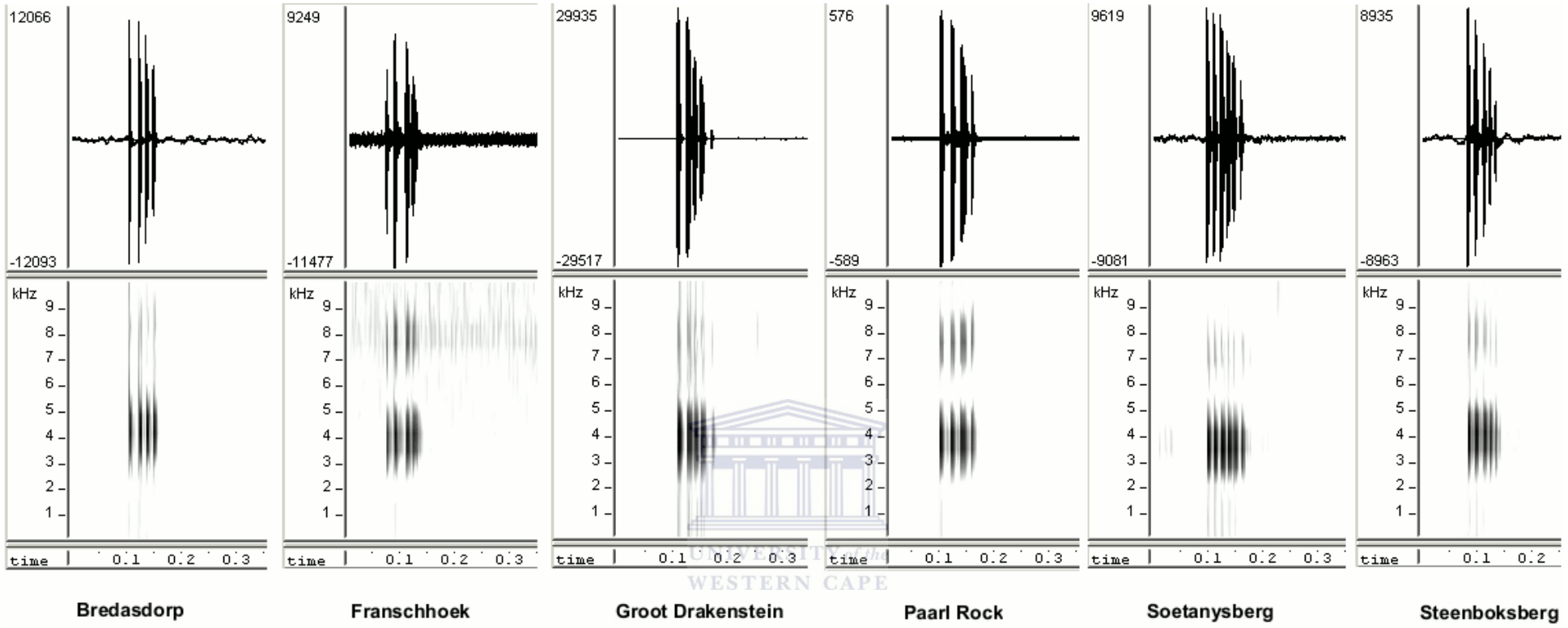

Figure 30. Advertisement calls of *A. villiersi* from various mountain ranges spanning the species distribution.

Call comparisons

Substantial variation in calls was found within individuals, between individuals, between populations from different mountain ranges and between different species. For the purposes of this study the variation within and between individuals is not addressed.

The Houwhoek population of *A. landdrosia* was analysed separately (as *A. landdrosia* "Houwhoek") to assess if treating it as a candidate species yielded significant advertisement call differences. This was done due to the audible differences observed when recording these calls in the field. Note that the pattern of significant differences between tables with *A. landdrosia* "Houwhoek" separate and tables with *A. landdrosia* "Houwhoek" subsumed in *A. landdrosia* may differ for species other than *A. landdrosia* "Houwhoek" and *A. landdrosia.* This is because the p-value correction (Holm's) applied to the pairwise Mann-Whitney tests depends on the entire family of comparisons which is different between the two scenarios.

The results of the dominant frequency comparison are presented in Tables 22 to 24, duration in Tables 25 to 27, note rate in Tables 28 to 30, notes per call in tables 31 to 33, pulse rate in Tables 35 to 37, pulses per call in Tables 38 to 40 and pulses per note in Tables 41 to 43.

Dominant frequency

Dominant frequency varies over a narrow band with average emphasised frequencies falling between 3500 and 4500 kHz. Within this range members of the Chirping clade had lower frequencies than members of the Clicking clade.

Kruskal-Wallis and ANOVA tests across mountain ranges and species were highly significant for all seven call variables (Table 22 and Table 24). This was evident whether or not the Houwhoek *A. landdrosia* population was included with the rest of *A. landdrosia*. Pairwise tests below identify the sources of the significant differences.

Abbreviations for mountain ranges and species names are shown in Table 21.

Table 21. Abbreviations used for mountain ranges and species.

Mountain range		Species	
Babilonstoring (*A. drewesii*)	BT	*A. sp.* A West	AW
Babilonstoring (*A. villiersi*)	BTV	*A. sp.* A East	AE
Caledon Swartberg	CS	*A. bicolor*	bi
Cape Point	CT	*A. drewesii*	dr
Constantiaberg	CB	*A. landdrosia* "Houwhoek"	hw
Du Toitskloof	DT	*A. sp.* B	B
Groenlandberg	GB	*A. sp.* C	C
Groot Drakenstein	GD	*A. landdrosia*	la
Grootwinterhoek	GW	*A. lightfooti*	li
Hottentots-Holland (*A. landdrosia*)	HH	*A. rugosa*	ru
Hottentots-Holland (*A. villiersi*)	HHV	*A. subvoce*	su
Houwhoek	HW	*A. villiersi*	vi
Klein Drakenstein	KD		
Kleinriviersberg	KR		
Kogelberg	KB		
Limietberg	LB		
Riviersonderendberg East	RSE		
Riviersonderendberg West	RSW		
Simonsberg	SB		
Hermanus (not a mountain)	(blank)		

Table 22. Pairwise Mann-Whitney comparisons of dominant frequency across mountain ranges. Cells are labelled 2 for $p < 0.001$, 1 for $p < 0.05$ and 0 for $p >= 0.05$.

	BT	BTV	CS	CT	CB	DT	GB	GD	GW	HH	HHV	HW	KD	KR	KB	LB	RSE	RSW	SB
BT																			
BTV	2																		
CS	2	0																	
CT	2	2	2																
CB	2	2	2	2															
DT	2	2	2	2	2														
GB	0	2	2	2	2	2													
GD	2	2	2	0	2	2	0												
GW	2	2	2	2	2	2	2	2											
HH	2	2	2	2	2	0	2	2	2										
HHV	0	2	2	2	2	2	0	2	2	2									
HW	2	2	2	2	2	2	2	2	2	2	2								
KD	2	2	2	2	2	0	2	2	2	0	2	2							
KR	2	2	2	2	2	2	2	2	2	2	2	1	2						
KB	2	2	2	2	2	2	2	2	2	2	2	2	2	0					
LB	2	2	2	2	2	0	2	2	2	0	2	2	1	0	1				
RSE	2	2	2	2	2	2	2	2	2	2	2	0	2	2	2	2			
RSW	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	0	2		
SB	2	2	2	2	2	1	2	2	2	1	2	2	1	0	0	0	2	0	

Table 23. Pairwise Mann-Whitney comparisons of dominant frequency across taxa. Cells are labelled 2 for p < 0.001, 1 for p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	hw	B	C	la	li	ru	su	vi
AW												
AE	2											
bi	0	2										
dr	2	0	2									
hw	2	1	2	0								
B	2	2	1	2	2							
C	0	2	1	2	2	2						
la	2	2	0	2	2	0	2					
li	2	2	2	2	2	2	2	2				
ru	2	2	2	2	2	2	2	2	2			
su	2	2	2	2	2	2	2	2	2	2		
vi	2	2	2	2	2	2	2	2	0	2	2	

Table 24. Pairwise Mann-Whitney comparisons of dominant frequency across taxa with the Houwhoek population considered part of *A. landdrosia*. Cells are labelled 2 for p < 0.001, 1 for p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	B	C	la	li	ru	su	vi
AW											
AE	2										
bi	0	2									
dr	2	0	2								
B	2	2	1	2							
C	0	2	2	2	2						
la	0	2	2	2	2	0					
li	2	2	2	2	2	2	2				
ru	2	2	2	2	2	2	2	2			
su	2	2	2	2	2	2	2	2	2		
vi	2	2	2	2	2	2	2	0	2	2	

Most pairwise comparisons were significantly different. Of particular interest were the differences between the populations of *A. sp.* A and also the differences between the Houwhoek population of *A. landdrosia* and other populations of *A. landdrosia*. There are clear population differences between the Riviersonderend populations of *A. sp.* A (candidate species AE and AW corresponding to mountain ranges RSE and RSW: eastern and western Riviersonderend mountains respectively).

The eastern clade, with the exception of the Twistwyk population (at the extreme east of the range), had lower dominant frequencies than the western clade (Table 7). Similarly there was a distinction between the Houwhoek population of *A. landdrosia* and other populations remainder of *A. landdrosia*.

Duration

Table 25. Pairwise Mann-Whitney comparisons of call duration across mountain ranges. Cells are labelled 2 for $p < 0.001$, 1 for $p < 0.05$ and 0 for $p >= 0.05$.

	BT	BTV	CS	CT	CB	DT	GB	GD	GW	HH	HHV	HW	KD	KR	KB	LB	RSE	RSW	SB
BT																			
BTV	2																		
CS	2	2																	
CT	2	2	2																
CB	2	2	2	2															
DT	1	2	2	2	2														
GB	2	2	2	2	2	1													
GD	2	0	2	2	2	2	2												
GW	1	2	2	2	2	2	2	2											
HH	0	2	2	2	2	2	2	2	2										
HHV	2	0	2	2	2	2	2	0	2	2									
HW	2	2	2	2	2	2	2	2	2	2	2								
KD	2	2	2	2	2	2	1	2	2	2	2	2							
KR	2	2	2	2	2	2	1	2	2	2	2	2	0						
KB	0	2	2	2	2	0	2	2	2	1	2	2	2	2					
LB	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2				
RSE	2	2	2	2	2	2	2	2	2	2	2	2	2	1	2	2			
RSW	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0		
SB	2	2	2	2	2	0	0	2	2	2	2	2	2	2	1	2	2	2	

Table 26. Pairwise Mann-Whitney comparisons of call duration across candidate species. Cells are labelled 2 for p < 0.001, 1 for p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	hw	B	C	la	li	ru	su	vi
AW												
AE	0											
bi	2	2										
dr	2	2	2									
hw	2	2	0	2								
B	2	2	2	1	2							
C	2	2	2	0	2	2						
la	2	2	2	1	2	2	0					
li	2	2	2	2	2	2	2	2				
ru	2	2	2	2	2	2	2	2	2			
su	2	2	2	2	2	2	2	2	2	2		
vi	2	2	2	2	2	2	2	2	2	2	2	

Table 27. Pairwise Mann-Whitney comparisons of call duration across candidate species with the Houwhoek population considered part of *A. landdrosia*. Cells are labelled 2 for p < 0.001, 1 for p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	B	C	la	li	ru	su	vi
AW											
AE	0										
bi	2	2									
dr	2	2	2								
B	2	2	2	2							
C	2	2	2	2	1						
la	2	2	2	2	2	1					
li	2	2	2	2	2	2	2				
ru	2	2	2	2	2	2	2	2			
su	2	2	2	2	2	2	2	2	2		
vi	2	2	2	2	2	2	2	2	2	2	

Note rate

Table 28. Pairwise Mann-Whitney comparisons of note rate across mountain ranges. Cells are labeled 2 for $p < 0.001$, 1 for $p < 0.05$ and 0 for $p >= 0.05$.

	BT	BTV	CS	CT	CB	DT	GB	GD	GW	HH	HHV	HW	KD	KR	KB	LB	RSE	RSW	SB
BT																			
BTV	2																		
CS	2	2																	
CT	2	2	2																
CB	2	2	2	2															
DT	2	2	2	2	2														
GB	0	2	2	2	2	2													
GD	2	0	2	2	2	2	2												
GW	2	2	2	2	2	2	2	2											
HH	2	2	2	2	2	2	2	2	2										
HHV	2	0	2	2	2	2	2	0	2	2									
HW	2	2	2	2	0	2	2	2	2	2	2								
KD	2	2	2	2	2	2	2	2	2	2	2	2							
KR	2	2	2	2	2	2	1	2	2	2	2	2	2						
KB	2	2	2	2	2	1	2	2	2	2	2	2	2	2					
LB	0	2	2	2	2	2	0	2	2	2	2	2	2	0	2				
RSE	2	2	2	2	2	0	2	2	2	1	2	2	2	2	1	2			
RSW	2	2	2	2	2	2	2	2	2	2	2	2	0	1	2	2	2		
SB	2	2	2	2	2	2	2	2	2	2	2	2	0	2	2	2	2	1	

Table 29. Pairwise Mann-Whitney comparisons of note rate across candidate species. Cells are labelled 2 for p < 0.001, 1 for p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	hw	B	C	la	li	ru	su	vi
AW												
AE	2											
bi	2	2										
dr	2	2	0									
hw	2	2	2	2								
B	2	2	2	2	2							
C	1	2	2	2	2	0						
la	1	2	2	2	2	2	2					
li	2	2	2	2	2	2	2	2				
ru	2	2	2	2	2	2	2	2	2			
su	2	2	2	2	2	2	2	2	2	2		
vi	2	2	2	2	0	2	2	2	2	2	2	

Table 30. Pairwise Mann-Whitney comparisons of note rate across candidate species with the Houwhoek population considered part of *A. landdrosia*. Cells are labelled 2 for p < 0.001, 1 for p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	B	C	la	li	ru	su	vi
AW											
AE	2										
bi	2	2									
dr	2	2	0								
B	2	2	2	2							
C	2	1	2	2	0						
la	0	2	0	2	2	2					
li	2	2	2	2	2	2	2				
ru	2	2	2	2	2	2	2	2			
su	2	2	2	2	2	2	2	2	2		
vi	2	2	2	2	2	2	2	2	2	2	

Notes per call

Table 31. Pairwise Mann-Whitney comparisons of notes per call across mountain ranges. Cells are labelled 2 for $p < 0.001$, 1 for $p < 0.05$ and 0 for $p >= 0.05$.

	BT	BTV	CS	CT	CB	DT	GB	GD	GW	HH	HHV	HW	KD	KR	KB	LB	RSE	RSW	SB
BT																			
BTV	2																		
CS	2	2																	
CT	2	0	2																
CB	2	0	2	0															
DT	2	2	2	2	2														
GB	2	2	2	2	2	2													
GD	2	0	2	0	0	2	2												
GW	2	2	2	2	2	2	2	2											
HH	2	2	2	2	2	1	2	2	2										
HHV	2	0	2	0	0	2	2	0	2	2									
HW	2	2	2	2	2	0	2	2	2	2	2								
KD	2	2	2	2	2	1	2	2	2	2	2	0							
KR	2	2	2	2	2	0	2	2	2	0	2	0	0						
KB	2	2	2	2	2	2	0	2	2	0	2	2	2	0					
LB	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2				
RSE	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1			
RSW	2	2	2	2	2	2	2	2	0	2	2	2	2	2	2	2	2		
SB	2	2	2	2	2	0	1	2	2	0	2	2	2	0	0	2	2	2	

Table 32. Pairwise Mann-Whitney comparisons of notes per call across candidate species. Cells are labelled 2 for p < 0.001, 1 for p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	hw	B	C	la	li	ru	su	vi
AW												
AE	2											
bi	1	2										
dr	2	2	2									
hw	2	2	2	2								
B	2	2	2	2	2							
C	2	2	2	2	1	2						
la	2	2	2	2	0	2	0					
li	2	2	2	2	2	2	2	2				
ru	2	2	2	2	2	2	2	2	2			
su	2	0	2	2	2	2	2	2	2	2		
vi	2	2	2	2	2	2	2	2	0	2	2	

Table 33. Pairwise Mann-Whitney comparisons of notes per call across candidate species with the Houwhoek population considered part of *A. landdrosia*. Cells are labelled 2 for p < 0.001, 1 for p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	B	C	la	li	ru	su	vi
AW											
AE	2										
bi	2	1									
dr	2	2	2								
B	2	2	2	2							
C	2	2	2	2	2						
la	2	2	2	2	2	1					
li	2	2	2	2	2	2	2				
ru	2	2	2	2	2	2	2	2			
su	0	2	2	2	2	2	2	2	2		
vi	2	2	2	2	2	2	2	0	2	2	

In some species of the Clicking clade the males may utter long trains of single click notes. This is particularly evident in *A. landdrosia* and *A. drewesii* but also occurs to a lesser extent in *A. sp.* C and *A. sp.* B and to a much lesser degree in *A. bicolor* and *A. subvoce*. This is reflected in the maximum number of notes per call (Table 34).

Table 34. Maximum number of notes per call.

Species	Maximum notes per call	Std. dev. notes per call
Arthroleptella villiersi	1	0.00
Arthroleptella lightfooti	1	0.00
Arthroleptella rugosa	5	0.74
Arthroleptella bicolor	10	1.25
Arthroleptella sp. A West	10	1.20
Arthroleptella sp. A East	12	1.21
Arthroleptella subvoce	14	1.41
Arthroleptella landdrosia	15	2.05
Arthroleptella sp. B	16	2.12
Arthroleptella sp. C	18	2.34
Arthroleptella drewesii	20	3.59
Arthroleptella landdrosia	27	2.54

It is difficult to determine the cause of the long click trains as it was impossible to observe calling males as they were completely obscured and were very sensitive to disturbance of surrounding vegetation. They cease calling upon sensing movement or noise nearby. The presence of a female appeared to lead to an increase in calling rate and reduction of sensitivity to disturbance on two occasions, one involving *A. villiersi* (which does not make clicks) and one involving *A. subvoce* in which males continued to call as I approached them for capture and in both cases a gravid female was found close to the calling males. From this very limited observation and the observation that long click trains occur erratically rather than continuously, I infer that the long click trains are probably not related to the proximity of females.

Pulse rate

Table 35. Pairwise Mann-Whitney comparisons of pulse rate across mountain ranges. Cells are labelled 2 for $p < 0.001$, 1 for $p < 0.05$ and 0 for $p >= 0.05$.

	BT	BTV	CS	CT	CB	DT	GB	GD	GW	HH	HHV	HW	KD	KR	KB	LB	RSE	RSW	SB
BT																			
BTV	2																		
CS	2	2																	
CT	2	0	2																
CB	2	2	2	2															
DT	2	2	2	2	2														
GB	2	2	2	2	2	2													
GD	2	0	2	0	2	2	2												
GW	2	2	2	2	2	2	2	2											
HH	2	2	2	2	2	2	2	2	0										
HHV	2	2	2	2	0	2	2	2	2	2									
HW	2	2	2	2	2	2	2	2	2	2	2								
KD	2	2	2	2	2	1	2	2	2	2	2	0							
KR	0	2	2	2	2	2	2	2	1	2	2	2	2						
KB	2	2	2	2	2	2	2	2	2	2	2	2	2	2					
LB	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2				
RSE	2	2	2	2	2	2	2	2	2	2	2	1	1	2	2	1			
RSW	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0		
SB	2	2	2	2	2	2	0	2	2	2	2	2	2	2	2	2	2	2	

Table 36. Pairwise Mann-Whitney comparisons of pulse rate across candidate species. Cells are labelled 2 for p < 0.001, 1 for p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	hw	B	C	la	li	ru	su	vi
AW												
AE	0											
bi	0	1										
dr	2	2	2									
hw	2	2	2	2								
B	2	2	2	2	1							
C	2	2	2	2	2	2						
la	2	2	2	2	2	2	2					
li	2	2	2	2	2	2	2	2				
ru	2	2	2	2	2	2	2	2	2			
su	2	2	2	2	2	2	2	2	2	2		
vi	2	2	2	2	2	2	2	2	0	2	2	

Table 37. Pairwise Mann-Whitney comparisons of pulse rate across candidate species with the Houwhoek population considered part of *A. landdrosia*. Cells are labelled 2 for p < 0.001, 1 for p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	B	C	la	li	ru	su	vi
AW											
AE	0										
bi	0	1									
dr	2	2	2								
B	2	2	2	2							
C	2	2	2	2	2						
la	2	2	2	2	2	2					
li	2	2	2	2	2	2	2				
ru	2	2	2	2	2	2	2	2			
su	2	2	2	2	2	2	2	2	2		
vi	2	2	2	2	2	2	2	0	2	2	

Pulses per call

Table 38. Pairwise Mann-Whitney comparisons of pulses per call across mountain ranges. Cells are labelled 2 for $p < 0.001$, 1 for $p < 0.05$ and 0 for $p >= 0.05$.

	BT	BTV	CS	CT	CB	DT	GB	GD	GW	HH	HHV	HW	KD	KR	KB	LB	RSE	RSW	SB
BT																			
BTV	2																		
CS	2	2																	
CT	2	2	2																
CB	2	2	2	2															
DT	2	2	2	2	2														
GB	0	2	2	2	2	2													
GD	2	0	2	2	2	2	2												
GW	2	2	2	2	2	2	2	2											
HH	2	2	2	2	2	2	2	2	0										
HHV	2	2	2	0	2	2	2	2	2	2									
HW	2	2	1	2	2	2	2	2	2	2	2								
KD	2	2	2	2	2	2	2	2	0	0	2	2							
KR	2	2	1	2	2	2	2	2	2	2	2	0	2						
KB	2	2	2	2	2	2	2	2	2	2	2	2	2	1					
LB	0	2	2	2	2	2	0	2	2	2	2	2	2	2	2				
RSE	2	2	2	2	2	2	2	2	0	0	2	2	0	2	2	2			
RSW	2	2	2	2	2	2	2	2	0	0	2	2	0	2	2	2	1		
SB	2	2	2	2	2	2	1	2	0	0	2	2	1	2	2	1	0	2	

Table 39. Pairwise Mann-Whitney comparisons of pulses per call across candidate species. Cells are labelled 2 for p < 0.001, 1 fro p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	hw	B	C	la	li	ru	su	vi
AW												
AE	1											
bi	2	2										
dr	2	2	2									
hw	2	2	2	2								
B	2	1	2	2	2							
C	2	2	2	2	1	2						
la	0	0	2	2	2	2	2					
li	2	2	2	2	2	2	2	2				
ru	2	2	2	2	2	2	2	2	2			
su	0	0	2	2	2	2	2	0	2	2		
vi	2	2	2	2	2	2	2	2	2	2	2	

Table 40. Pairwise Mann-Whitney comparisons of pulses per call across candidate species with the Houwhoek population considered part of *A. landdrosia*. Cells are labelled 2 for p < 0.001, 1 for p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	B	C	la	li	ru	su	vi
AW											
AE	1										
bi	2	2									
dr	2	2	2								
B	1	2	2	2							
C	2	2	2	2	2						
la	2	2	1	2	2	2					
li	2	2	2	2	2	2	2				
ru	2	2	2	2	2	2	2	2			
su	0	0	2	2	2	2	2	2	2		
vi	2	2	2	2	2	2	2	2	2	2	

Pulses per note

Table 41. Pairwise Mann-Whitney comparisons of pulses per note across mountain ranges. Cells are labelled 2 for p < 0.001, 1 for p < 0.05 and 0 for p >= 0.05.

	BT	BTV	CS	CT	CB	DT	GB	GD	GW	HH	HHV	HW	KD	KR	KB	LB	RSE	RSW	SB
BT																			
BTV	2																		
CS	2	2																	
CT	2	2	2																
CB	2	2	2	2															
DT	2	2	2	2	2														
GB	2	2	2	2	2	2													
GD	2	0	2	2	2	2	2												
GW	2	2	0	2	2	2	2	2											
HH	2	2	2	2	2	2	2	2	2										
HHV	2	2	2	0	2	2	2	2	2	2									
HW	2	2	2	2	2	2	2	2	2	2	2								
KD	2	2	2	2	2	1	2	2	2	2	2	2							
KR	2	2	2	2	2	2	2	2	2	2	2	0	2						
KB	2	2	2	2	2	2	2	2	2	2	2	2	2	2					
LB	2	2	1	2	2	0	2	2	2	2	2	2	1	2	2				
RSE	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2			
RSW	2	2	0	2	2	2	2	2	0	2	2	2	2	2	2	2	2		
SB	2	2	2	2	2	2	2	2	2	0	2	2	2	2	2	2	2	2	

Table 42. Pairwise Mann-Whitney comparisons of pulses per note across candidate species. Cells are labelled 2 for p < 0.001, 1 fro p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	hw	B	C	la	li	ru	su	vi
AW												
AE	2											
bi	2	2										
dr	2	2	2									
hw	2	2	2	2								
B	2	2	0	2	2							
C	2	2	2	2	0	2						
la	2	2	2	2	2	2	2					
li	2	2	2	2	2	2	2	2				
ru	0	2	1	2	2	2	2	2	2			
su	0	2	2	2	2	2	2	2	2	0		
vi	2	2	2	2	2	2	2	2	2	2	2	

Table 43. Pairwise Mann-Whitney comparisons of pulses per note across candidate species with the Houwhoek population considered part of *A. landdrosia*. Cells are labelled 2 for p < 0.001, 1 for p < 0.05 and 0 for p >= 0.05.

	AW	AE	bi	dr	B	C	la	li	ru	su	vi
AW											
AE	2										
bi	2	2									
dr	2	2	2								
B	2	2	0	2							
C	2	2	2	2	2						
la	2	2	2	2	2	2					
li	2	2	2	2	2	2	2				
ru	0	2	1	2	2	2	2	2			
su	0	2	2	2	2	2	2	2	0		
vi	2	2	2	2	2	2	2	2	2	2	

Arthroleptella landdrosia has more pulses per note, especially notes comprising more than 2 pulses. *Arthroleptella sp.* C does not have notes consisting of three or more pulses at the beginning of call that *A. landdrosia* often does. *Arthroleptella villiersi* from Soetanysberg had more pulses per note than other populations of *A. villiersi* or *A. lightfooti*.

Other call differences

Although aggressive calls were not formally analysed in this study, some differences were immediately apparent from *Arthroleptella sp.* C has a typical *Arthroleptella* aggressive call but no similar call has been heard uttered by *A. landdrosia*. Aggressive calls in this genus consist of a series of very closely spaced notes (Figure 31). Aggressive calls have not been recorded for *A. lightfooti* and *A. villiersi* although the sister species to this pair, *A. rugosa*, did emit aggressive calls.

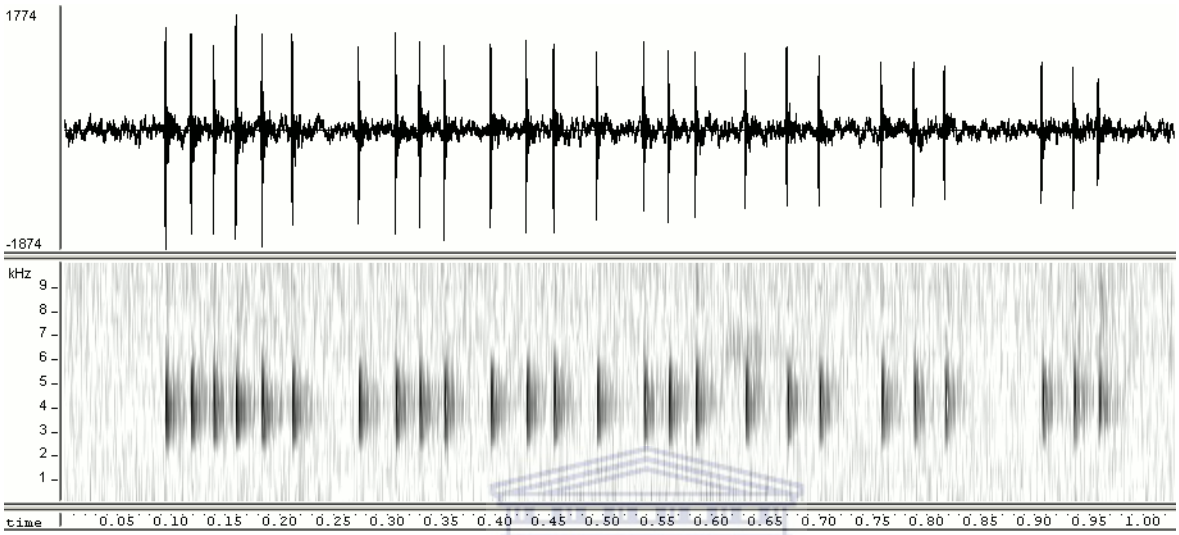

Figure 31. Wave form graph (above) and spectrogram (below) of an aggressive call from an *Arthroleptella subvoce* from the Grootwinterhoek Wilderness Area.

3.3 Summary of statistical analyses of call measurements

Almost all comparisons of the measured call variables show significant differences. As we do not currently know which of these variables are used by *Arthroleptella* in mate choice and hence species recognition, it is not possible to weight these variables according to their importance in species recognition. However, they were all treated as equal and the number of significantly different variables counted for each comparison across mountain ranges and species. These summaries are shown in Tables 44 to 46.

Table 44. Summary of number of call variables with significant differences from pairwise Mann-Whitney comparisons across mountain ranges.

	BT	CW	CT	CB	DT	GB	GD	GW	HH	HW	KD	KR	KB	LB	RS	SB
BT																
CW	7															
CT	7	7														
CB	7	7	7													
DT	7	7	7	7												
GB	7	7	7	7	7											
GD	7	7	7	7	7	7										
GW	7	7	7	7	7	7	7									
HH	7	7	7	7	7	7	7	7								
HW	7	7	7	7	7	7	7	7	7							
KD	7	7	7	7	5	7	7	7	7	7						
KR	7	7	7	7	7	7	7	7	7	7	7					
KB	7	7	7	7	7	7	7	7	6	7	7	7				
LB	7	7	7	7	7	7	7	7	7	7	7	6	7			
RS	7	7	7	7	7	7	7	7	7	7	7	7	7	6		
SB	7	7	7	7	7	7	7	7	7	7	6	7	7	6	7	

Table 45. Summary of number of call variables with significant differences from pairwise Mann-Whitney comparisons across candidate species.

	AW	AE	bi	dr	hw	B	C	la	li	ru	su	vi
AW												
AE	5											
bi	7	7										
dr	7	7	6									
hw	7	7	6	7								
B	7	7	6	7	7							
C	7	7	7	6	6	6						
la	6	6	7	7	6	7	6					
li	7	7	7	7	7	7	7	7				
ru	7	7	7	7	7	7	7	7	7			
su	6	5	7	7	7	7	7	6	7	6		
vi	7	7	7	7	6	7	7	7	5	7	7	

Table 46. Summary of number of call variables with significant differences from pairwise Mann-Whitney comparisons across candidate species with the Houwhoek populations considered part of *A. landdrosia*.

	AW	AE	bi	dr	B	C	la	li	ru	su	vi
AW											
AE	5										
bi	7	7									
dr	7	7	6								
B	7	7	6	7							
C	7	7	7	7	6						
la	7	7	7	7	7	7					
li	7	7	7	7	7	7	7				
ru	7	7	7	7	7	7	7	7			
su	6	6	7	7	7	7	7	7	6		
vi	7	7	7	7	7	7	7	6	7	7	

3.4 Phylogenetics

3.4.1 PCR and sequencing results

Extraction results

Chelex extractions yielded unpredictable results with DNA frequently not amplifying in PCR reactions and also generally produced lower DNA concentrations. Both the standard and modified phenol-chloroform yielded reliable and good quality DNA in the range of 22 to 276 ng/µl.

Alignment

Sequence alignments of 16S sequences were all unambiguous and straightforward. No sequences required manual alignment or excision of any sections.

Sequencing Results

All sequences used in this study have been or will be deposited in GenBank.

Table 47. Approximate gross length of amplified sequences.

Gene	Base pairs sequenced
12S	890
16S	550
RAG-1a	900
RAG-1b	500
Rag-2	450
Rhodopsin	330
Tyrosinase Precursor	450

Mitochondrial fragments

12S

A total of 54 sequences were obtained for the 12S fragment. In an alignment of 881 bp, 173 characters were variable, of which 38 were parsimony informative. Uncorrected p-distances (expressed as a percentage) varied from 0 to 10.9 % within *Arthroleptella*. For the placement of the genus *Arthroleptella* within the Pyxicephalidae, an alignment of 1 251 sites had 492 variable sites of which 285 were parsimony informative. This data set had uncorrected p distances varying from 9 to 21 %.

16S

A total of 76 sequences were obtained for the 16S fragment. An alignment of 516 bp contained 67 variable characters of which 62 were parsimony informative. Uncorrected p-distances varied from 0 to 7 % within *Arthroleptella*. In the data set comparing *Arthroleptella* to other pyxicephalid genera 186 sites were variable, of which 95 were parsimony informative with genetic distances of 6 to 20.1 %.

Nuclear fragments

Rhodopsin

Rhodopsin sequences were obtained for representative specimens of each of the clades identified by the mitochondrial 16S analysis. There was very little variation among *Arthroleptella* rhodopsin sequences with only five variable positions in a sequence length of 317 bp, of which only two were parsimony informative (n=21 individuals). The maximum uncorrected p-distance was 0.68 %. For the reason that rhodopsin contributes so little to elucidating relationships within the genus *Arthroleptella* it was excluded from analysis of species relationships. The rhodopsin gene fragment was however very useful in positioning the genus *Arthroleptella* in relation to other genera in the family Pyxicephalidae and to higher level relationships as previously found by Van der Meijden *et al*. (2005) and Frost *et al*. (2006).

Rag-1

Two contiguous fragments of Rag-1 were sequenced for the family phylogeny. Only the first fragment was used for analyses within *Arthroleptella*. An alignment of the first fragment using 845 bp contained 142 variable sites of which 68 were parsimony informative. A concatenation of both fragments made up 1 304 bp of which 377 were variable and 148 were parsimony informative. The concatenated Rag-1 sequences had uncorrected p-distances of between 3.3 % and 13.9 % across all pyxicephalid genera examined, and the first fragment distances varied from 0 to 8.1 % within *Arthroleptella* (31 sequences). Rag-1 proved to have useful variation at resolving genera but did not contribute much to the resolution of species. This result was expected due to the generally slower rate of mutation in nuclear genes relative to mitochondrial genes (e.g. Hoegg *et al*. 2004). However, in a study by Smith *et al.* (2007) using Rag-1, it showed the greatest degree of variation of the nuclear markers used.

Rag-2

A 736 bp fragment of Rag-2 was aligned for the family phylogeny. There were 277 variable characters and 111 were parsimony informative. Rag-2 sequences had uncorrected p-distances of

between 4 % and 18.8 % across all the pyxicephalid genera examined. This fragment was not used for the *Arthroleptella* phylogeny and its utility for this purpose was not established in this study.

Tyrosinase precursor

The tyrosinase precursor fragment yielded about 521 bp and had 22 variable sites of which only 6 were parsimony informative. Variation ranged from 0 to 2.1 % within *Arthroleptella*. The lack of variation in this gene precluded its inclusion for *Arthroleptella* phylogenies but it was useful in higher level analyses where it yielded 165 variable sites, of which 75 were parsimony informative, from an alignment of 473 bp. These sequences showed uncorrected p-distances from 4.5 % to 18.2 %.

Gene fragments not successfully amplified

For the future benefit of researchers working on this group, I list various gene fragments and associated primer pairs which did not amplify or provide reliable or useful PCR products or sequences. Various primer pairs were used to amplify a section of the sodium dehydrogenase subunit 2 (ND2) mitochondrial gene. These included the following primer pairs: VMet and VTrp (Cunningham & Cherry 2000),Vmet2 and VTrp (Cunningham & Cherry 2004); VGF1 and VWR1 (unpublished primer pair designed by M.J. Cunningham, University of the Free State, South Africa). The primer pair H4419 (Macey *et al.* 1997) and L5551 (Macey *et al.* 2001) and the primer pair H4419 and VWR1 generally failed to amplify reliably but did occasionally produce PCR product. However, the sequences were of insufficient quality for analysis. Amplification and sequencing of ND2 sequences were abandoned after many unsatisfactory attempts. This failure is discussed further in Chapter 4 in the section on future research.

Mitochondrial cytochrome b was amplified using the primer pair MNCN-Glu F and Amp-P10 R (San Mauro *et al.* 2004). Four sequences were obtained using this primer pair but the success rate was very low. The sequences obtained for this fragment were not used in any of the analyses.

The prolactin receptor gene (PRLR) primers published by Townsend *et al.* (2008) were tried but did not yield any product. The same primer pair was tested on reptile tissue and yielded high quality PCR product. This indicates that although this primer pair was designed for a wide spectrum of vertebrates it does not work on the genus *Arthroleptella*.

The primer pair FIB-B17U2 and FIB-B17L (Prychitko & Moore 1997) for beta fibrinogen was used in a PCR reaction and did amplify but yielded low concentration product. This gene fragment was

not used in any subsequent analyses. However, this primer pair may still be useful if yields can be increased by PCR optimization.

3.4.2 Nucleotide evolution model test results

Table 48. Results of the jModelTest multimodel inference tests of nucleotide substitution models. The tests are based on Akaike Information Criterion corrected for small samples (AICc).

Gene fragment	Best model (AICc)	Akaike weight
RAG-1	TrN+G	0.2936
RAG-2	HKY+G	0.2334
Tyrosinase Precursor	TIM2ef+G	0.3727
Rhodopsin	HKY+G	0.0912
12S	GTR+G	0.676
16S	GTR+G	0.5631
Nu (all nuclear fragments)	HKY+I+G	0.9342
Mt (all mitochondrial fragments)	GTR+G	0.676

The results of the jModelTest multimodel inference tests consistently selected the general time reversible (GTR) model with gamma-distributed rate variation (GTR+G) for the mitochondrial gene fragments 12S and 16S, both individually and when concatenated (Table 48). The best model selected for Rag-2 and Rhodopsin was the Hasegawa *et al.* (1985) model with gamma-distributed rate variation (HKY+G). For Rag-1 the best selected model was the Tamura & Nei (1993) model with gamma-distributed rate variation (TrN+G). The model selected for the Tyrosinase precursor was the TIM2ef with gamma-distributed rate variation. For a combined set of all the nuclear fragments, the best model was the Hasegawa *et al.* (1985) model with gamma-distributed rate variation plus a proportion of invariable sites (HKY+I+G). Akaike weights for the best models varied considerably (by an order of magnitude) for the different gene fragments.

Based on these results, the models of nucleotide substitution used in the Mr Bayes programme was the GTR model with a gamma distribution of rate variation across sites. Additionally, a GTR model with a proportion of invariant sites and with a gamma distribution of rate variation across sites was also run based on the sequence alignments which showed many conserved sites particularly in the nuclear sequences.

Effect of nucleotide substitution model

Only two models of nucleotide evolution available in Mr Bayes were indicated as appropriate from prior model fit testing viz. GTR + I + G and GTR + G. Changing the underlying nucleotide

evolution model from GTR + I + G to GTR + G had no effect (topology or branch lengths) on the resulting phylogenetic trees in MR Bayes.

3.4.3 Family phylogeny

A Bayesian phylogeny of all the southern African Pyxicephalidae yields a sister relationship between *Arthroleptella* and *Natalobatrachus* (see Figure 32). This relationship is very strongly supported by a bootstrap value of 100 under maximum parsimony and a Bayesian posterior probability of 1.

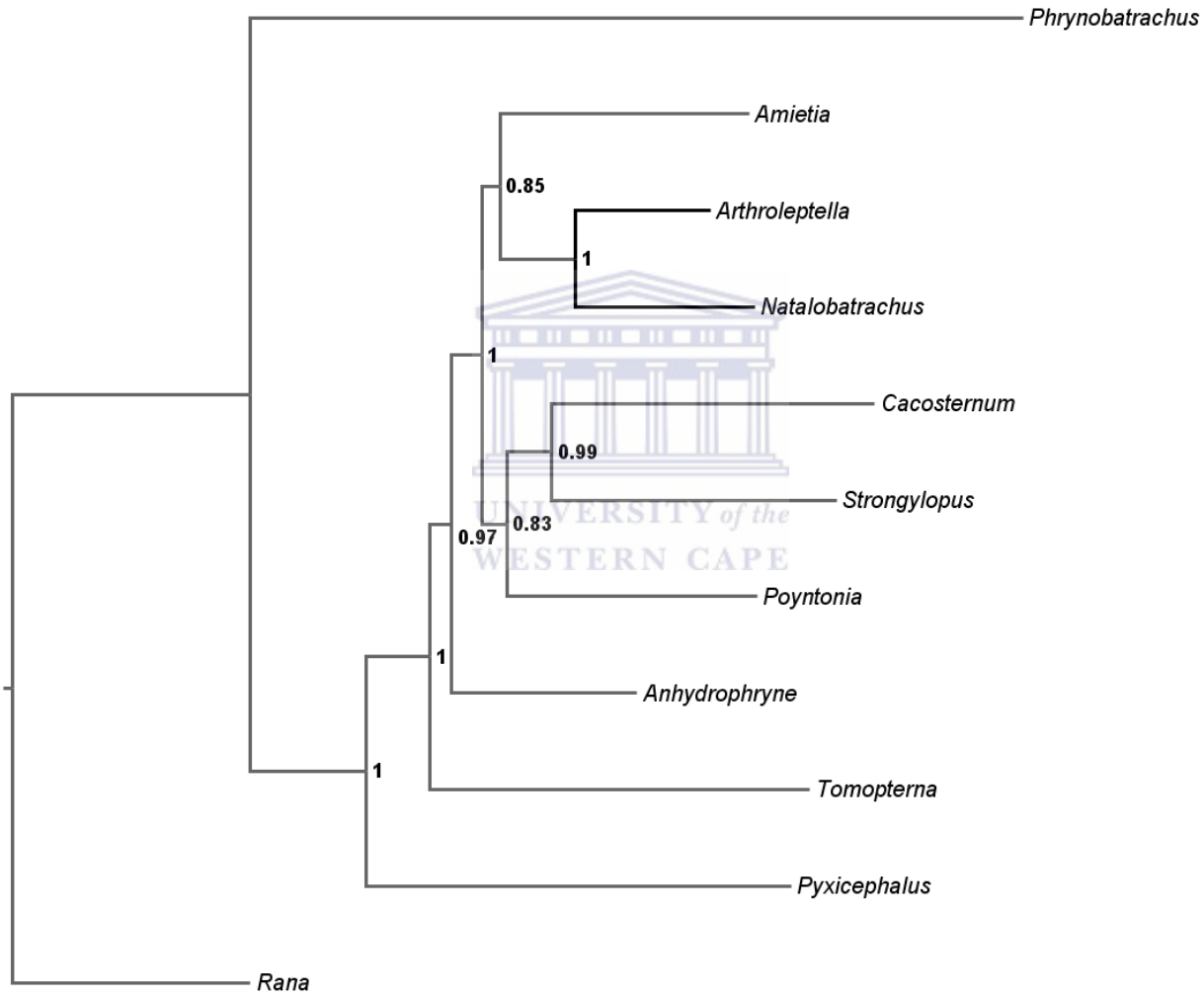

Figure 32. Family level phylogeny constructed using Mr Bayes based on 6 gene fragments (12S, 16S, Rag-1, Rag-2, Tyrosinase precursor and Rhodopsin) of the Pyxicephalidae showing the relative position of *Arthroleptella and Natalobatrachus* (black lines).

The placement of the *Arthroleptella* and *Natalobatrachus* pair relative to *Amietia*, *Cacosternum* and *Strongylopus* is less well supported. A more extensive taxon sampling of the pyxicephalids positions the *Arthroleptella* and *Natalobatrachus* pair similarly (Van der Meijden *et al.* in press).

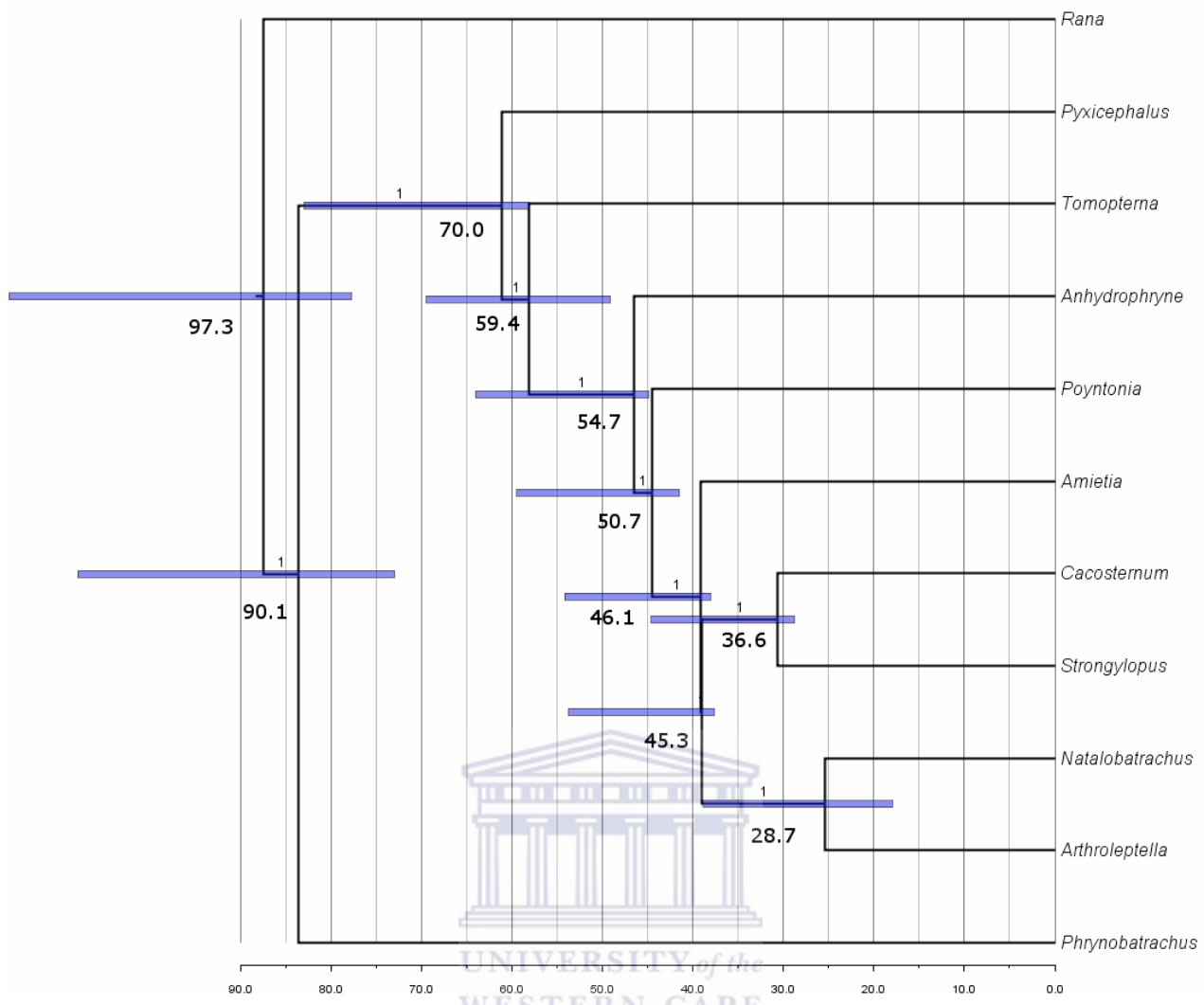

Figure 33. Timing of branch splits (Ma) based on 6 gene fragments (12S, 16S, Rag-1, Rag-2, Tyrosinase precursor and Rhodopsin) superimposed on a maximum clade credibility tree. The horizontal bars (lilac) represent 95% highest posterior density bounds.

The results of a Bayesian coalescent method for estimating genus coalescences with a relaxed molecular clock is shown in Figure 30. The posterior probabilities calculated by the programme BEAST are one (maximum possible value) for all clades. The maximum clade credibility tree produced by BEAST is similar to the Mr Bayes tree except for the placement of *Poyntonia*. The Clade coalescence times for the Pyxicephalidae are in good agreement with the times estimated by Van der Meijden *et al*. 2005 as expected (see Table 3). The clade coalescing *Pyxicephalus, Cacosternum* and *Tomopterna* as estimated by Roelants *et al.* (2007) also compares reasonably well with the estimate presented here (difference of < 10 Ma). The clade coalescing *Natalobatrachus* and *Arthroleptella* is dated to 28.7 Ma (95% highest posterior density bounds from 38.9 to 18 Ma).

3.4.4 Genus phylogeny

Estimation of the *Arthroleptella* phylogeny was done with both individual gene sequences and with concatenated gene sequences. The results of the individual and concatenated gene sequences are

presented first followed by a description of the clade interpretations in relation to species trees. The approach taken in this study is to run separate gene analyses and compare the gene trees manually (Ronquist & Deans 2010).

Mitochondrial phylogeny

Individual gene tree reconstructions are provided separately to expose the relative roles of each gene fragment to the combined gene phylogeny. Results of Bayesian analyses are presented followed by a brief comparison of phylogenetic trees derived from maximum parsimony and direct optimisation techniques.

Table 49. List of locality abbreviations used in specimen labels on gene trees.

Abbreviation	Locality	Abbreviation	Locality
AFT	Aasfontein	HLB	Helderberg
AGL	Agulhas	HWH	Houwhoek
AMZ	Amanzi	JAK	Jonaskop
AVGK	Aasvogelberg	JNK	Jonkershoek
BBT	Babilonstoring	KGB	Kogelberg
BDB	Bredasdorpberg	KLB	Koelberg
BNK	Bainskloof	KNB	Kanonberg
BTB	Betty's Bay	LDK	Landdroskop
CAL	Caedon	OBP	Observation Peak
CCP	Cecilia Plantation	PAR	Paarl Mountain
CPT	Cape Town	RSB	Rusbos
DGB	Die Galgberg	SGT	Sneeugat
DTK	Du Toitskloof	SLM	Silvermine
FHP	Franschhoek Pass	SMB	Simonsberg
FNK	Fernkloof	STBB	Stellenboschberg
FZK	Fizantakraal	SWK	Swartboskloof
GLB	Groenlandberg	TBMT	Table Mountain
GRYT	Greyton	TWK	Twistniet
GTDK	Groot Drakenstein	UNK	Unknown
GWH	Groot Winterhoek	WTV	Waterval N.R.
HGK	Hagelkraal	ZHK	Zachariashoek

12S

The 12S fragment provided a useful amount of genetic resolution and was largely congruent with the 16S phylogeny. The degree of genetic divergence as measured by uncorrected genetic distance ranged from 0 % to 11 % across all sampled sites. Posterior probabilities were generally high (>0.9).

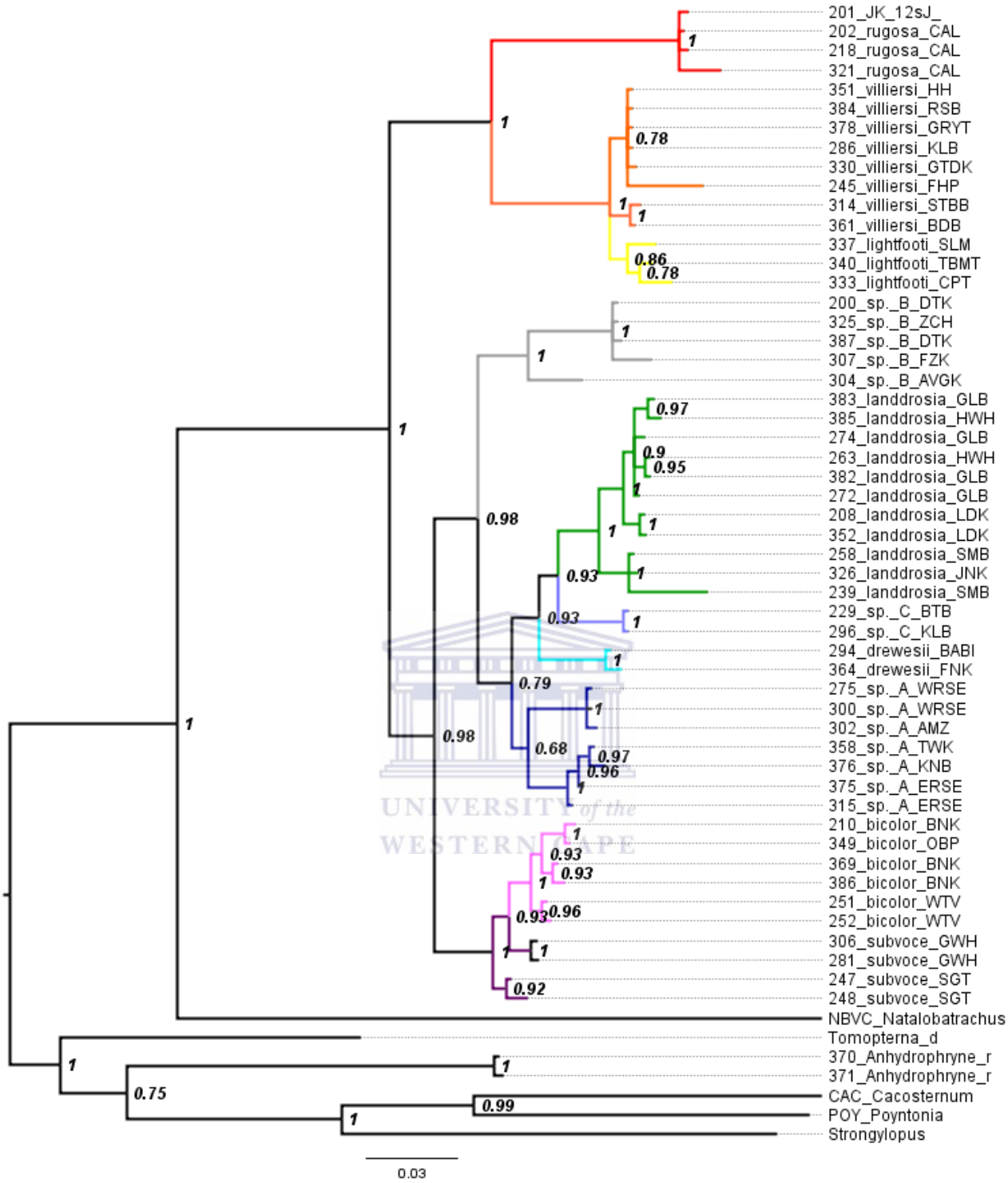

Figure 34. Consensus phylogram derived from Bayesian analysis of the12S fragment. Clades are coloured as follows: *A. rugosa* red; *A. villiersi* – orange; *A. lightfooti* – yellow; *A. sp.* B – grey; *A. landdrosia* – green; *A. sp.* C – cornflower blue; *A. drewesii* – bright blue; *A. sp.* A – dark blue; *A. bicolor* – pink; *A. subvoce* – purple. Location abbreviations for the specimens are listed in Table 49.

16S phylogeny

The 16S ribosomal mitochondrial gene fragment proved to be very useful in terms of both the genetic resolution it provided relative to the spatial and temporal scale of this investigation and the correspondence with the geography of the populations sampled. This fragment was very easily and

reliably sequenced. Signal strength was good and no problems were encountered in alignment. Forward and reverse sequences were complementary. Posterior probabilities were mostly high (>0.9).

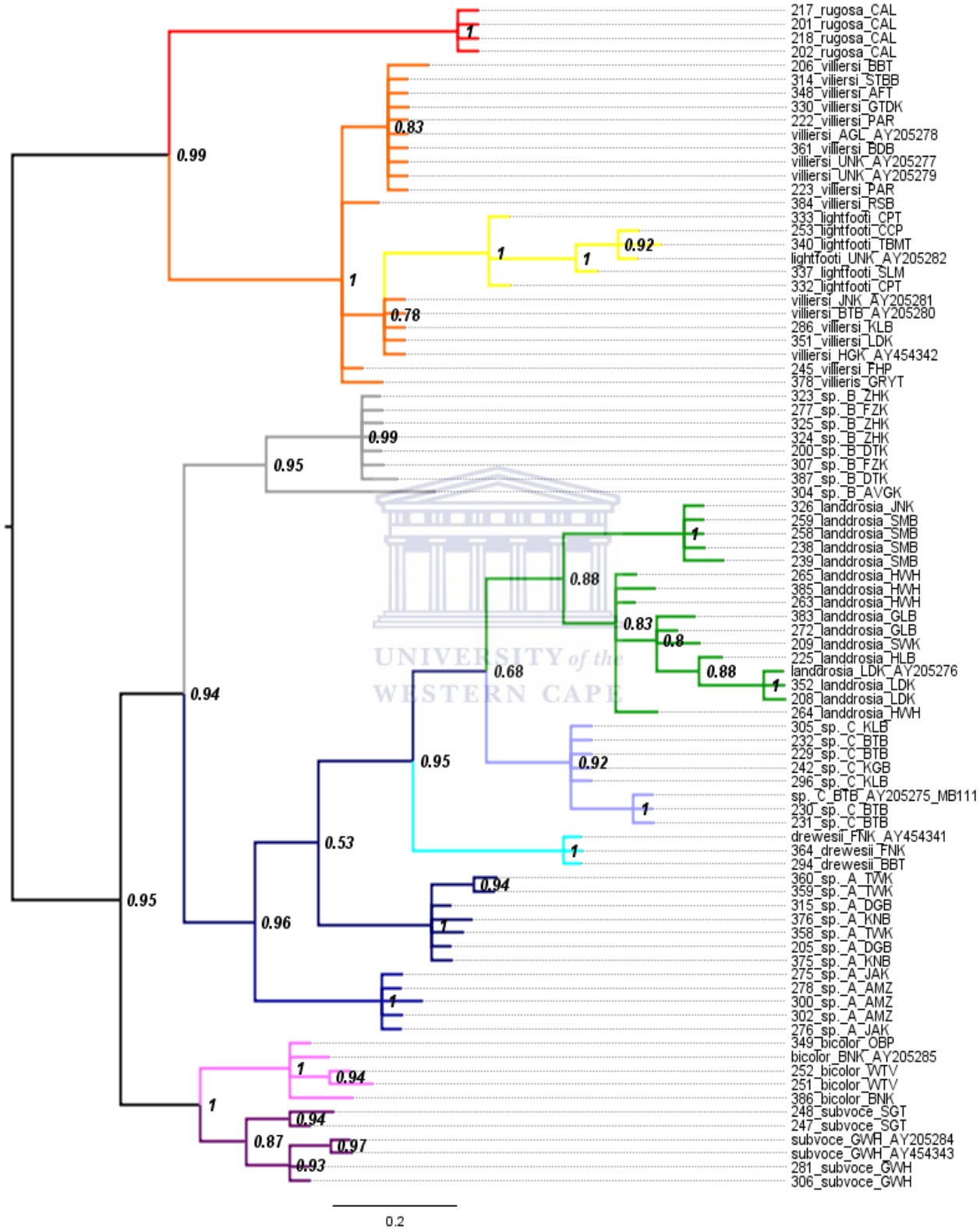

Figure 35. Consensus phylogram derived from Bayesian analysis of the 16S fragment.

16S & 12S combined phylogeny

Combining the 12S and 16S sequences resulted in a phylogeny with generally very well supported clades (most posterior probabilities = 1).

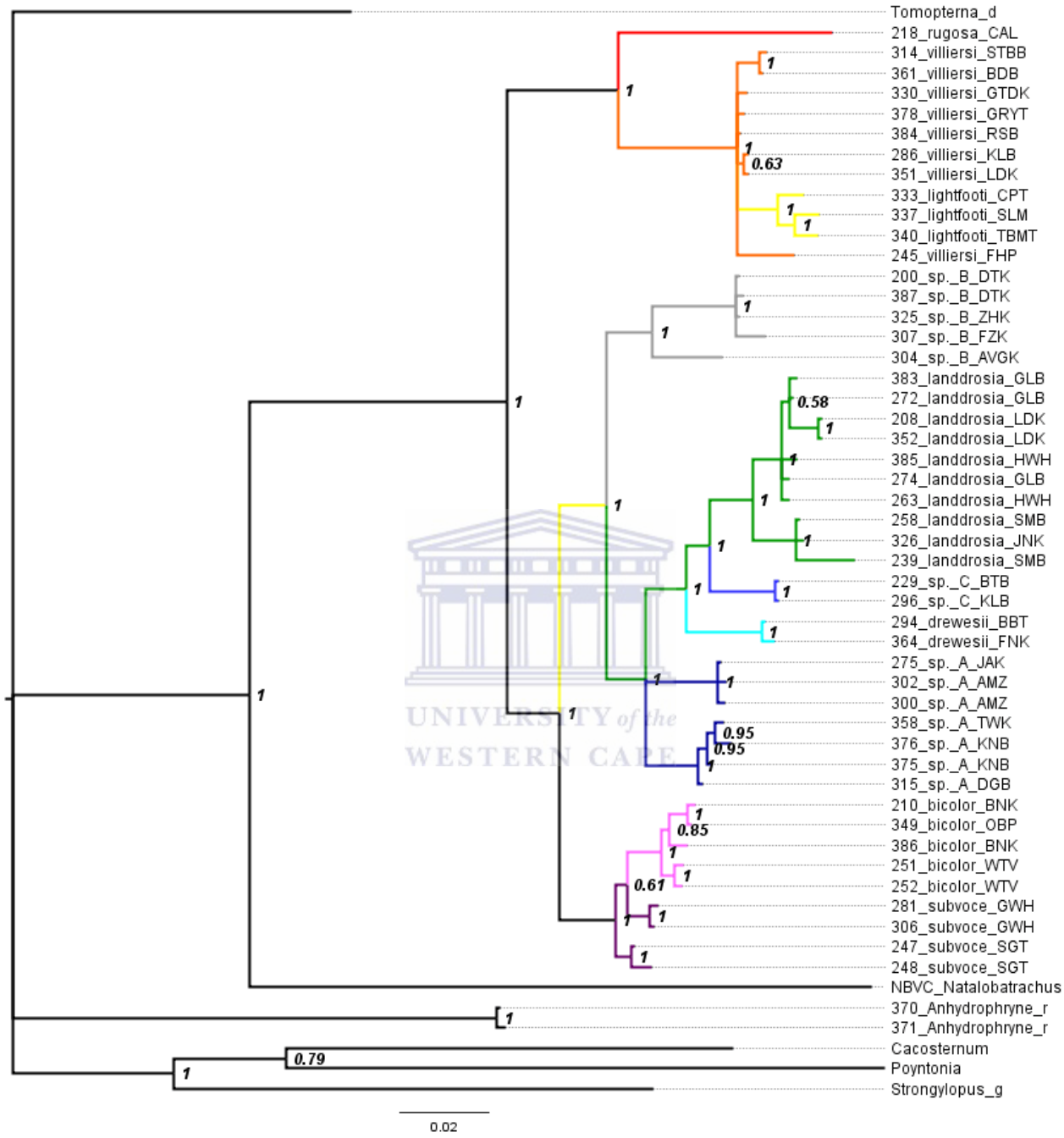

Figure 36. Consensus phylogram derived from Bayesian analysis of both the 12S and16S fragments.

Nuclear phylogeny

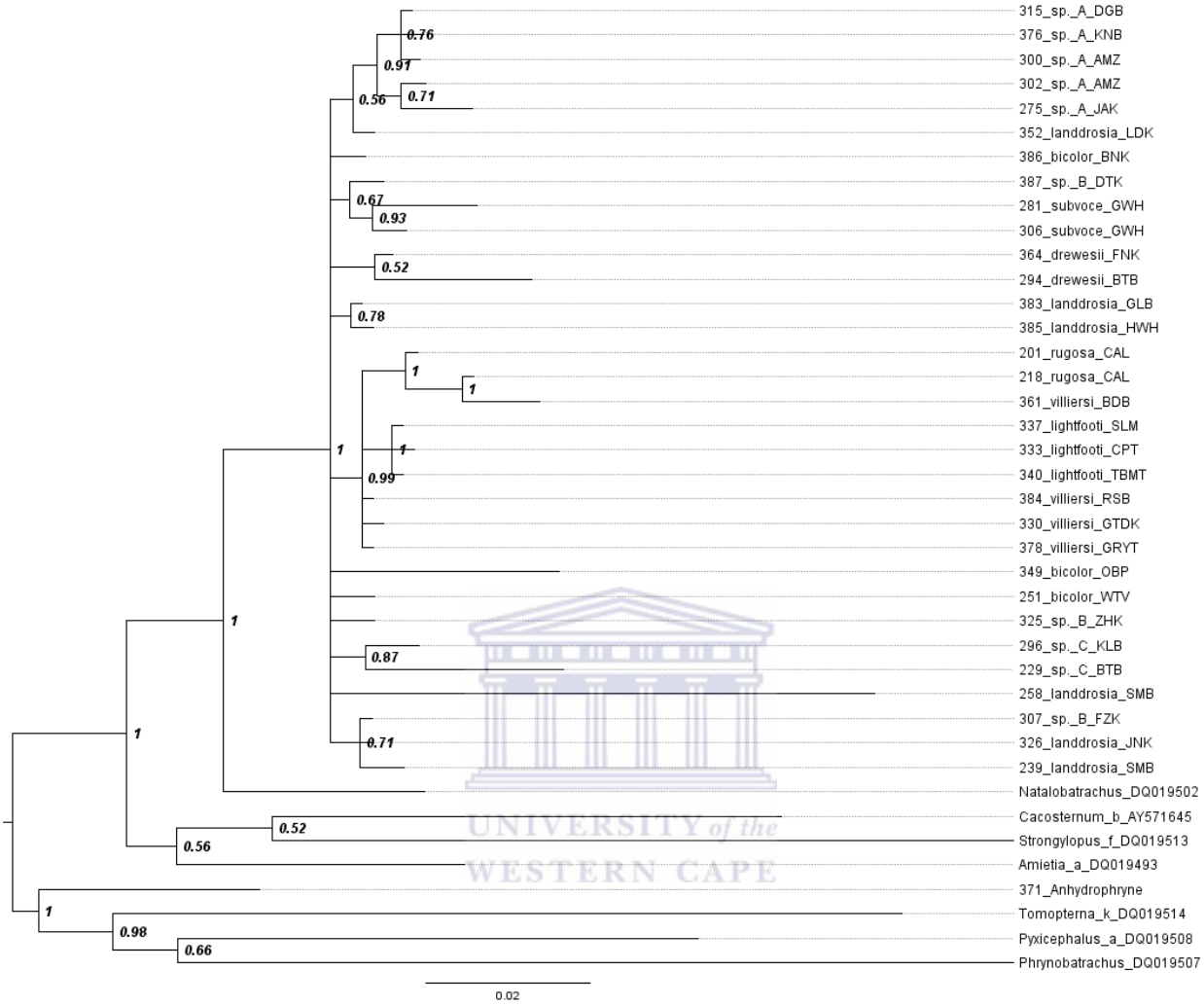

Figure 37. Consensus phylogram derived from Bayesian analysis of the Rag-1 fragment displaying multiple polyphyly and poor posterior probabilities on many branches. (Clade colour coding omitted due to lack of resolution).

The phylogenies of *Arthroleptella* derived from the Rag-1, Rhodopsin and Tyrosinase precursor sequences were poorly resolved and did not correspond well with either the mitochondrial phylogenies or the geography of the sampled populations (see Figure 36). Posterior probabilities were low for many clades. However, all the nuclear sequences used in conjunction with the mitochondrial sequences were useful in the construction of the family phylogeny (see section above on family phylogeny). Of note, however, in all three Rag-1 tree construction methods is the grouping together of *A. lightfooti* samples (within a polyphyletic *A. villiersi* clade) and a monophyletic *A. subvoce* clade.

Mt & Nu combined phylogeny

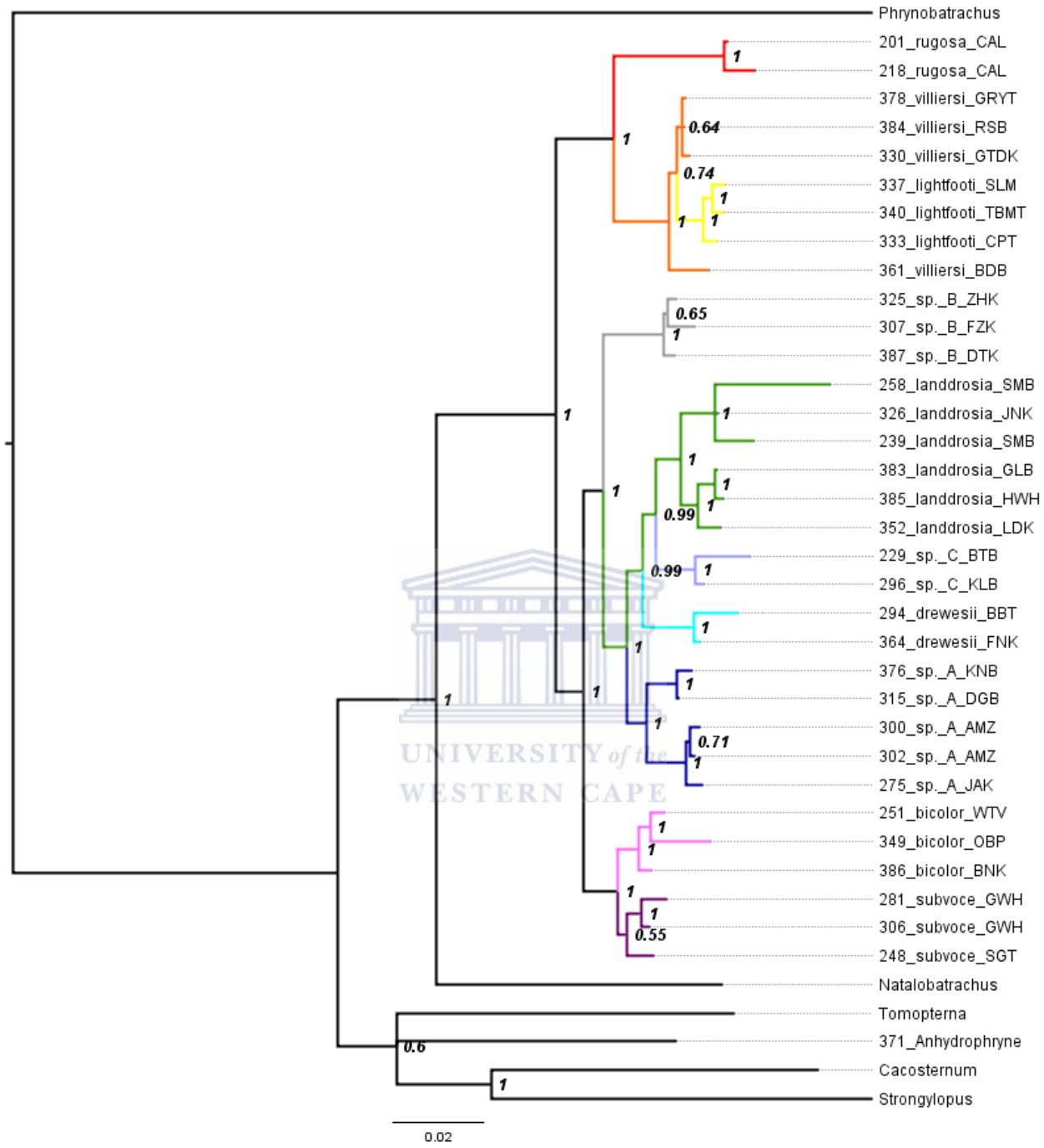

Figure 38. Consensus phylogram derived from Bayesian analysis of the combined Rag-1, 12S and 16S fragments.

The Bayesian analysis of the combined mitochochondrial and Rag-1 yielded a well-resolved phylogeny largely consistent with the Bayesian mitochondrial phylogeny (Figure 36) although the position of the two clades of *A. sp*. A is better resolved in the combined mitochochondrial and Rag-1 tree (Figure 38).

The generation of the mitochondrial and combined sequence phylogenies allowed the identification of the following clades. Bayesian, MP and POY phylogenies always produce a basal split within *Arthroleptella*. These are termed the Chirping and Clicking clades based on the characteristic call types produced by members of each of these clades and are defined as follows:

Chirping Clade

The Chirping clade comprises *A. lightfooti, A. villiersi* and *A. rugosa*.

Lightfooti-villiersi Clade

Within the Chirping clade there is *A. rugosa* and a clade containing the 'sister' taxa *A. lightfooti* and *A. villiersi*. The sister relationship as described previously (Dawood & Channing 2000; Turner & Channing 2008) is not always retrieved with *A. lightfooti* occasionally nested within *A. villiersi* in some of the gene trees. The individuals falling into either *A. lightfooti* or *A. villiersi* are referred to as the Lightfooti-villiersi clade.

Clicking Clade

The Clicking clade is a complex assemblage of several described species and genetically distinct populations. The clade contains the following taxa: *A. sp.* A West, *A. sp.* A East, *A. bicolor, A. drewesii, A. sp.* B, *A. sp.* C, *A. landdrosia* and *A. subvoce*.

Landdrosia Clade

Within the Clicking clade there is a generally well supported but diverse clade containing *A. sp.* A West, *A. sp.* A East, *A. drewesii, A. sp.* C, *A. landdrosia*. Within the Landdrosia clade there is good support for *A. drewesii* and topotypical *A. landdrosia*. The geographically coherent *A. sp.* A West and *A. sp.* A East are dealt with separately below. *A. sp.* C occupies a sister relationship to *A. landdrosia*. *Arthroleptella. landdrosia* as currently construed contains well supported clades within it with noticeable differences between the Hottentots-Holland, Jonkershoek, Simonsberg, Groenlandberg and Houwhoek Mountains.

Riviersonderend Clade

Within the Clicking clade there are two genetic groupings from the Riviersonderend Mountains. They are referred to as *A. sp.* A West from the western part of the range and *A. sp.* A East from the eastern part. The arrangement of these two entities varies from being arranged as sister taxa (Figure 34) to *A. sp.* A East being basal to the Clicking clade with *A. sp.* A West basal to the Landdrosia clade (Figure 35).

A. Sp. B Clade

There is a generally well-supported clade in the base of the Clicking group which contains a taxon referred to as *A. sp.* B. Individuals from the Du Toitskloof Mountains are always grouped strongly together with the single individual from Aasvogelberg near Villiersdorp basal in this grouping.

Bicolor clade

A. bicolor and *A. subvoce* are sister taxa (Turner *et al.* 2004) and are almost always present together in a single clade. Within this clade there are two alternative arrangements. In the first arrangement (Figure 35) *A. bicolor* and *A. subvoce* form reciprocally monophyletic clades. In the second arrangement the Groot Winterhoek subvoce clade is grouped with the Sneeugat *A. subvoce* clade. This is the cause of the low bootstrap value (51) on the node joining the Groot Winterhoek *A. subvoce* clade *to A. bicolor* clade as there is a high frequency of both alternatives. The Sneeugat clade causes paraphyly in the *A. bicolor* and *A. subvoce* clades in the 12S and combined 12S and 16S gene trees (Figure 34 and Figure 36).

Bayesian phylogeny results

Mitochondrial fragments provided good terminal resolution but poor basal resolution particularly in maximum parsimony and direct optimisation using POY. The nuclear marker Rag-1 provided good basal resolution but poor terminal resolution.

Although the resolution provided by the Rag-1 fragment was poor at the species level it did yield monophyletic *A. subvoce* and *A. lightfooti* clades which was unexpected given the polytomies and paraphyletic clades yielded by the mitochondrial fragments.

When the mitochondrial (12S and 16S) sequences were used in conjunction with the Rag-1 data sets the resulting phylogenies were topologically consistent with the mitochondrial phylogeny (Figure 36 and Figure 37). However, terminal resolution was not as good and bootstrap values were lower (Figure 37). This result is due to the swamping effect of the greater number of informative characters in the mitochondrial sequences used (197 vs. 68).

Comparison to other phylogenetic tree methods

In general the MP and POY analyses resulted in less resolution with more polytomies. This result is expected for MP based on the averaging effect of constructing consensus trees in which some information is necessarily lost. It is unclear why POY produced this result. POY analyses searching

for more than 1000 generations (5000) did not result in better support or more resolved trees. The main discrepancies between the methods are as follows:

12S: MP placed *A. rugosa* in a basal polytomy with *A. lightfooti* and *A. villiersi* (Figure 39). POY placed all species within *Arthroleptella* except the Chirping clade and the sister pair *A. bicolor* and *A. subvoce* in a basal polytomy (Figure 40).

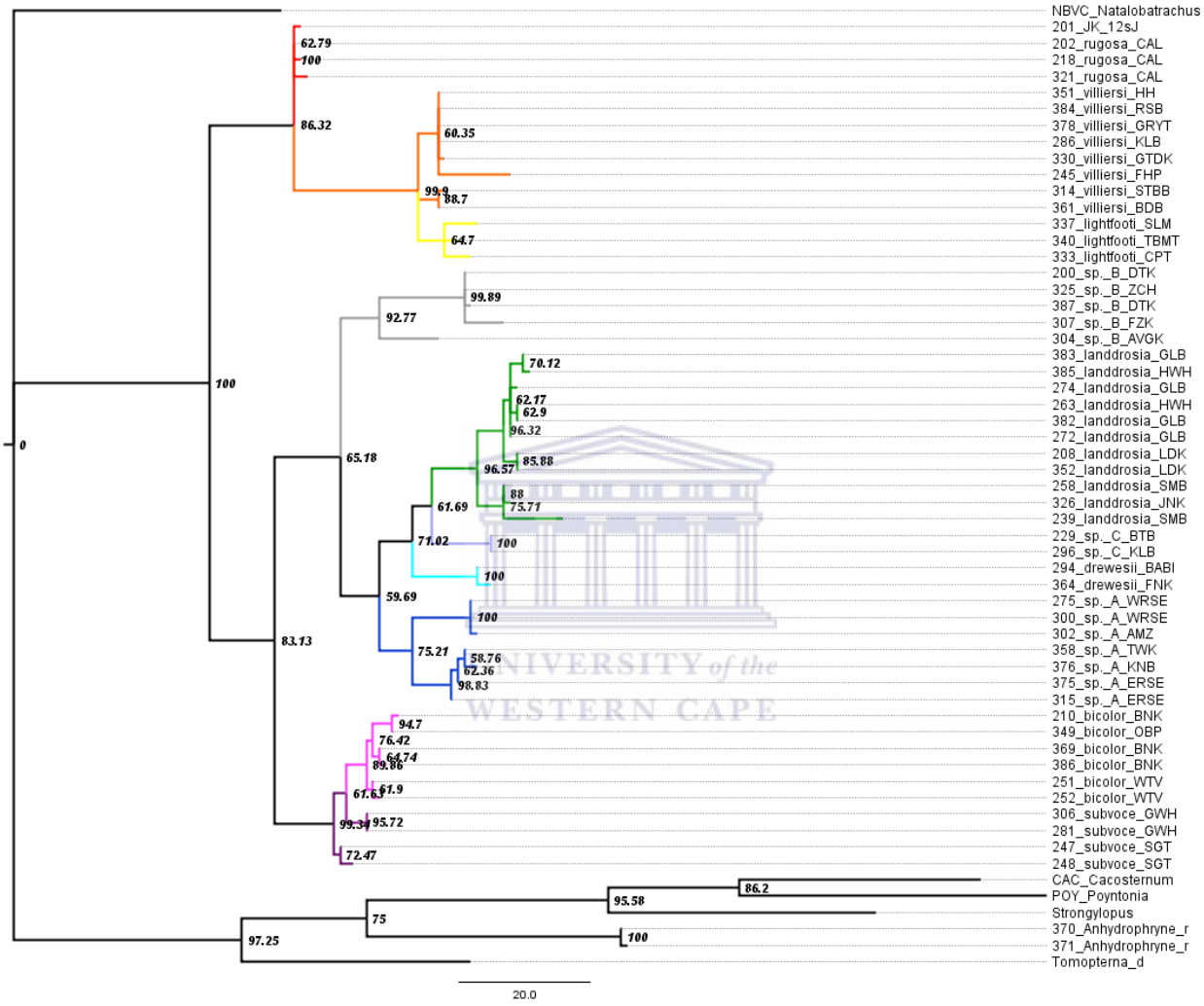

Figure 39. Consensus phylogram derived from parsimony analysis of the12S fragment.

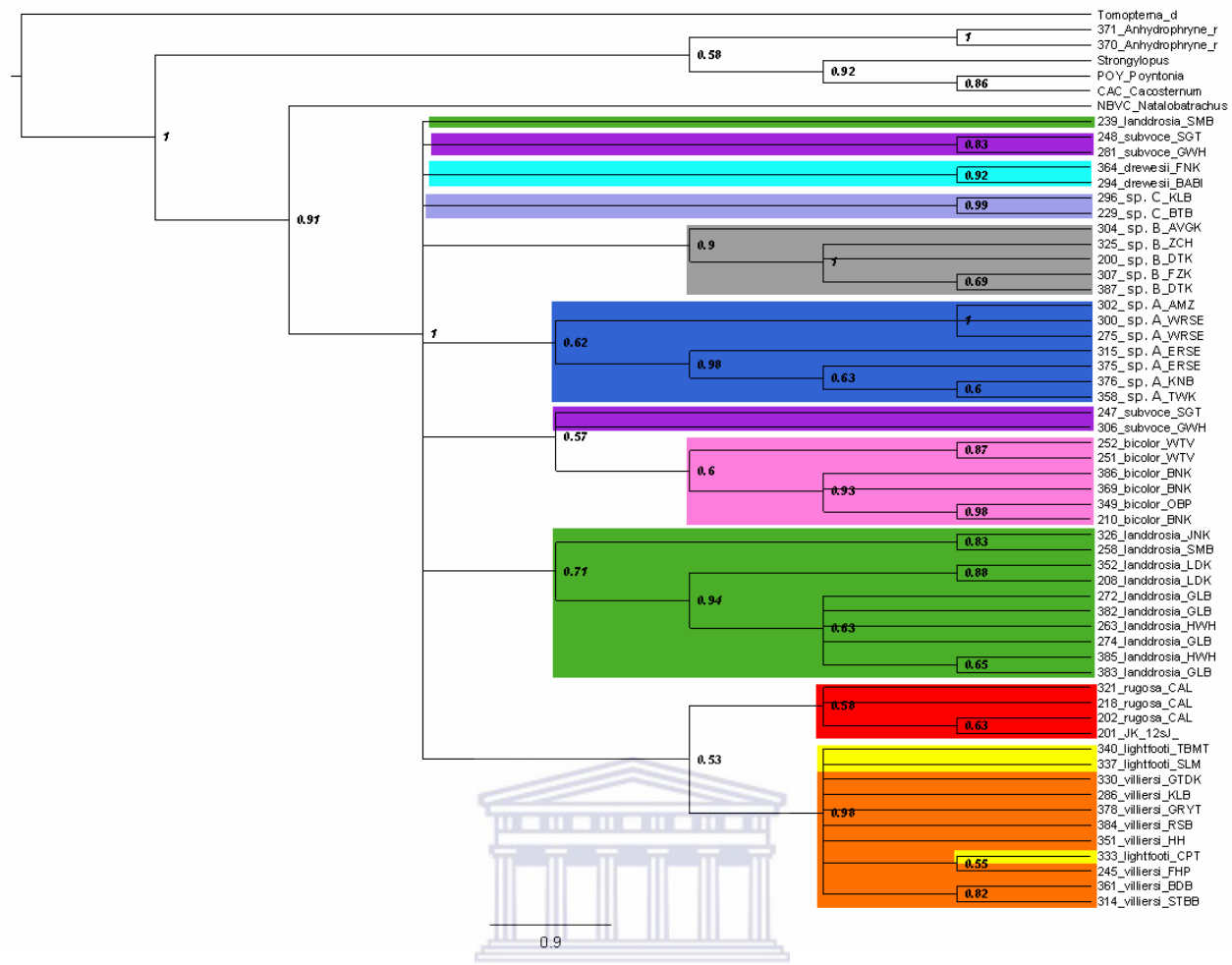

Figure 40. Consensus phylogram derived from direct optimisation analysis of the12S fragment.

16S: MP returned a phylogeny with all species within *Arthroleptella*, except the Chirping clade and the sister pair *A. bicolor* and *A. subvoce*, in a basal polytomy (Figure 41). POY yielded the same arrangement as MP for the 12S gene except that POY retrieved *A. lightfooti* as monophyletic with respect to *A. villiersi* (Figure 42). Both methods returned reciprocally monophyletic *A. bicolor* and *A. subvoce* clades.

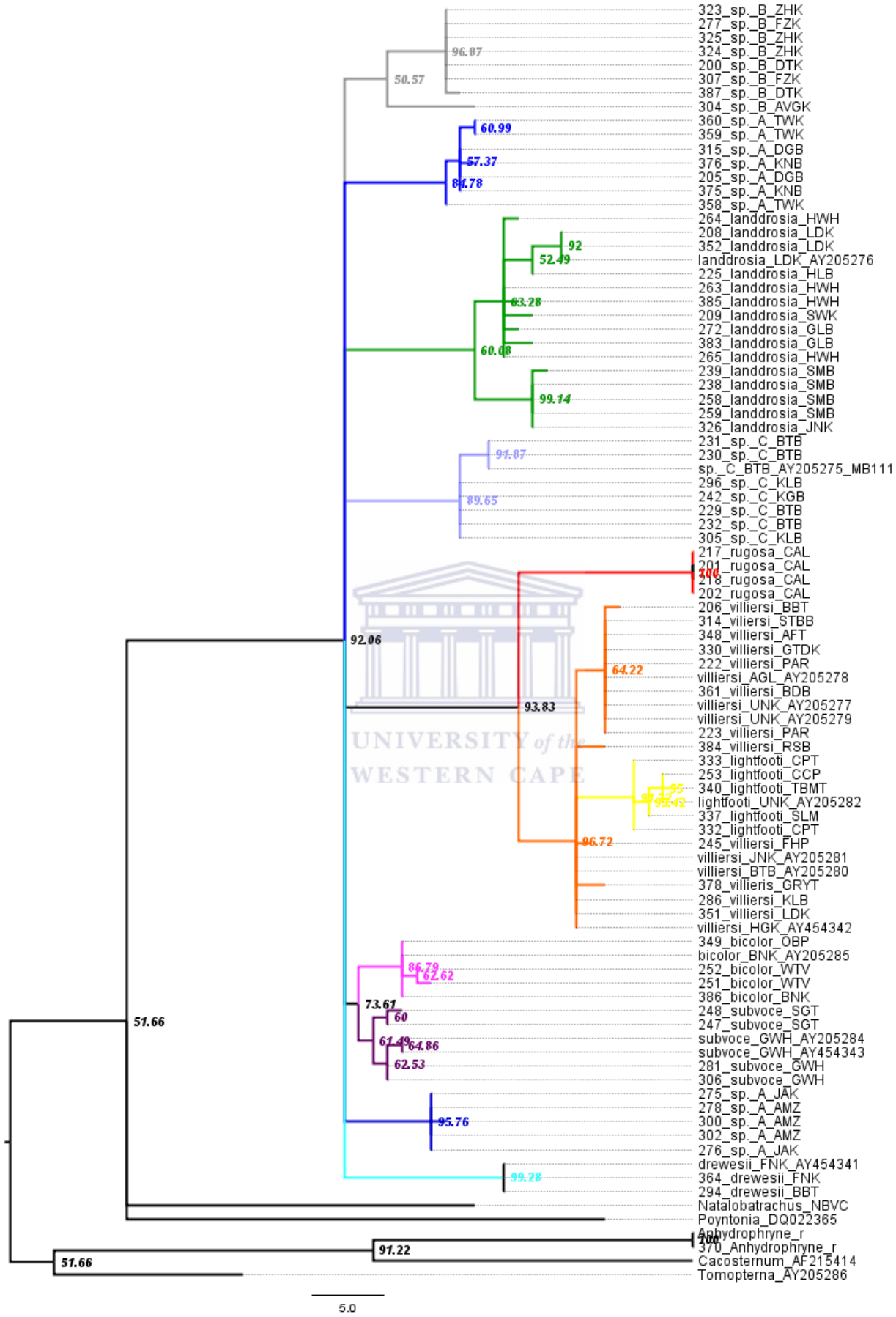

Figure 41. Consensus phylogram derived from parsimony analysis of 16S fragment.

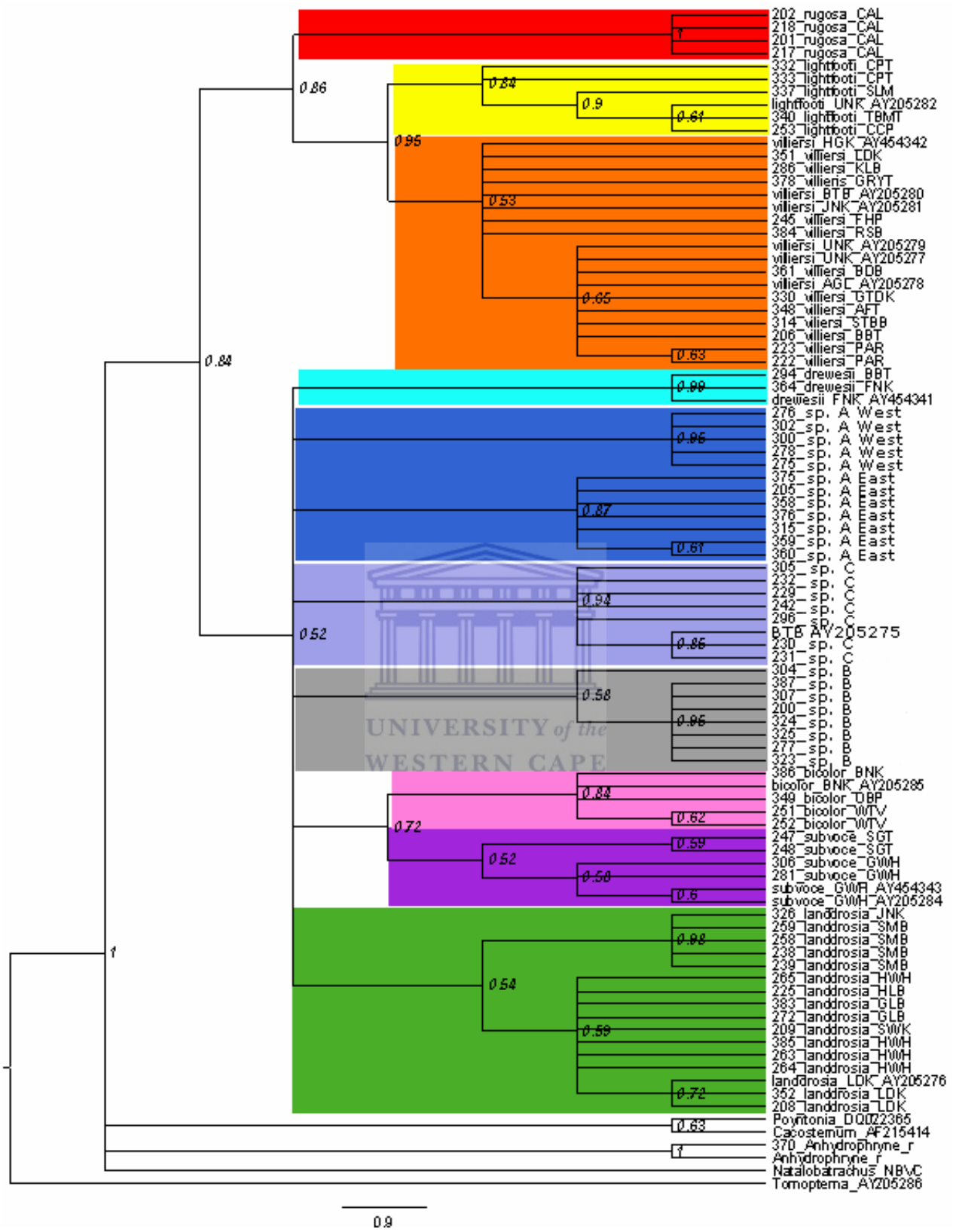

Figure 42. Consensus phylogram derived from direct optimisation analysis of the 16S fragment.

Combined 12S and 16S: MP and POY yielded topologies virtually identical to the Bayesian analysis for the combined 12S and 16S genes with only minor differences in basal positions of outgroups. The arrangement of *A. bicolor* and *A. subvoce* in the MP tree (Figure 43) matched the arrangement in the Bayesian 12S phylogram but the POY tree had the Sneeugat population of *A. subvoce* in a basal polytomy with the remaining *A. subvoce* and *A. bicolor* (Figure 44).

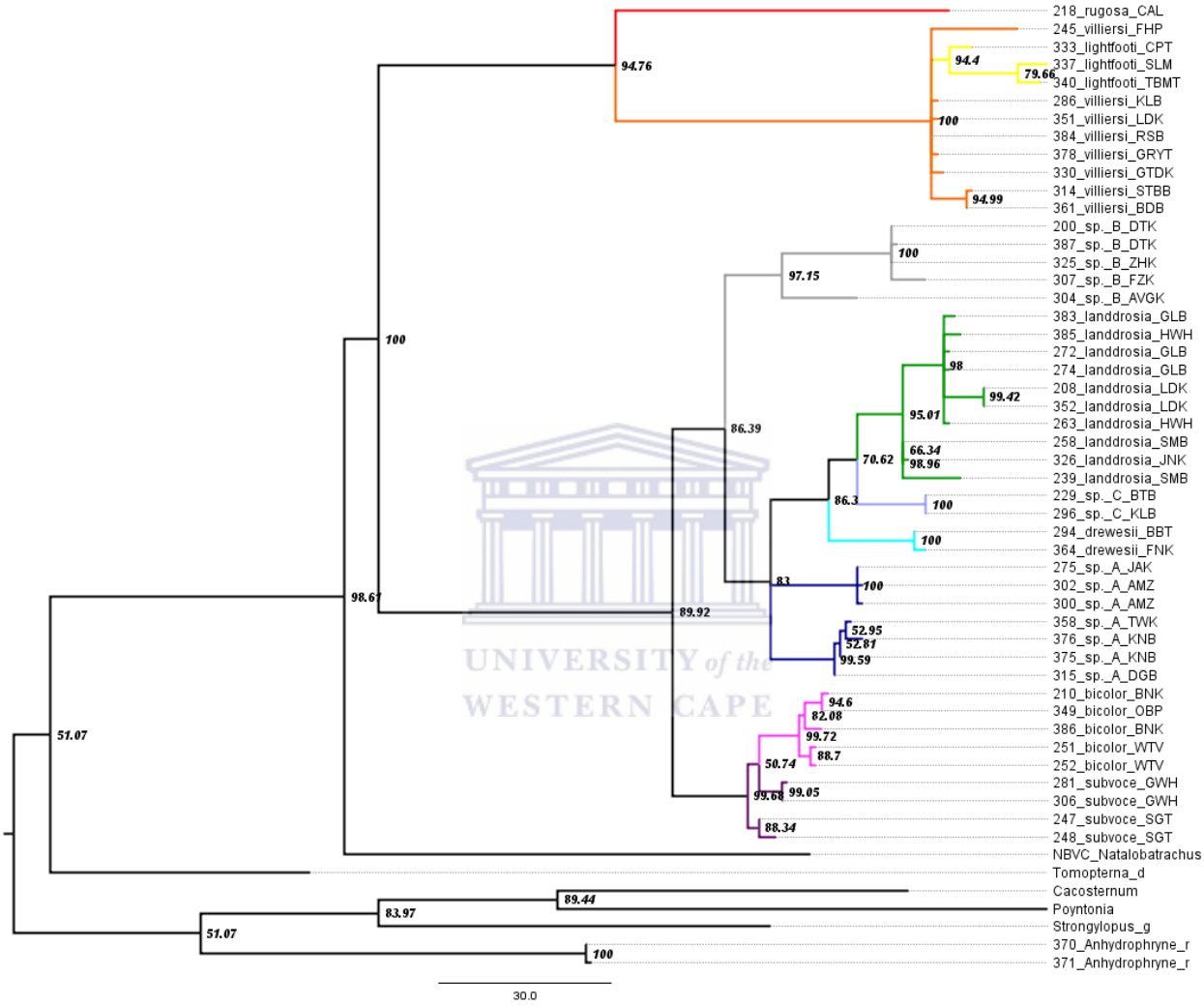

Figure 43. Consensus phylogram derived from parsimony analysis of both the 12S and16S fragments.

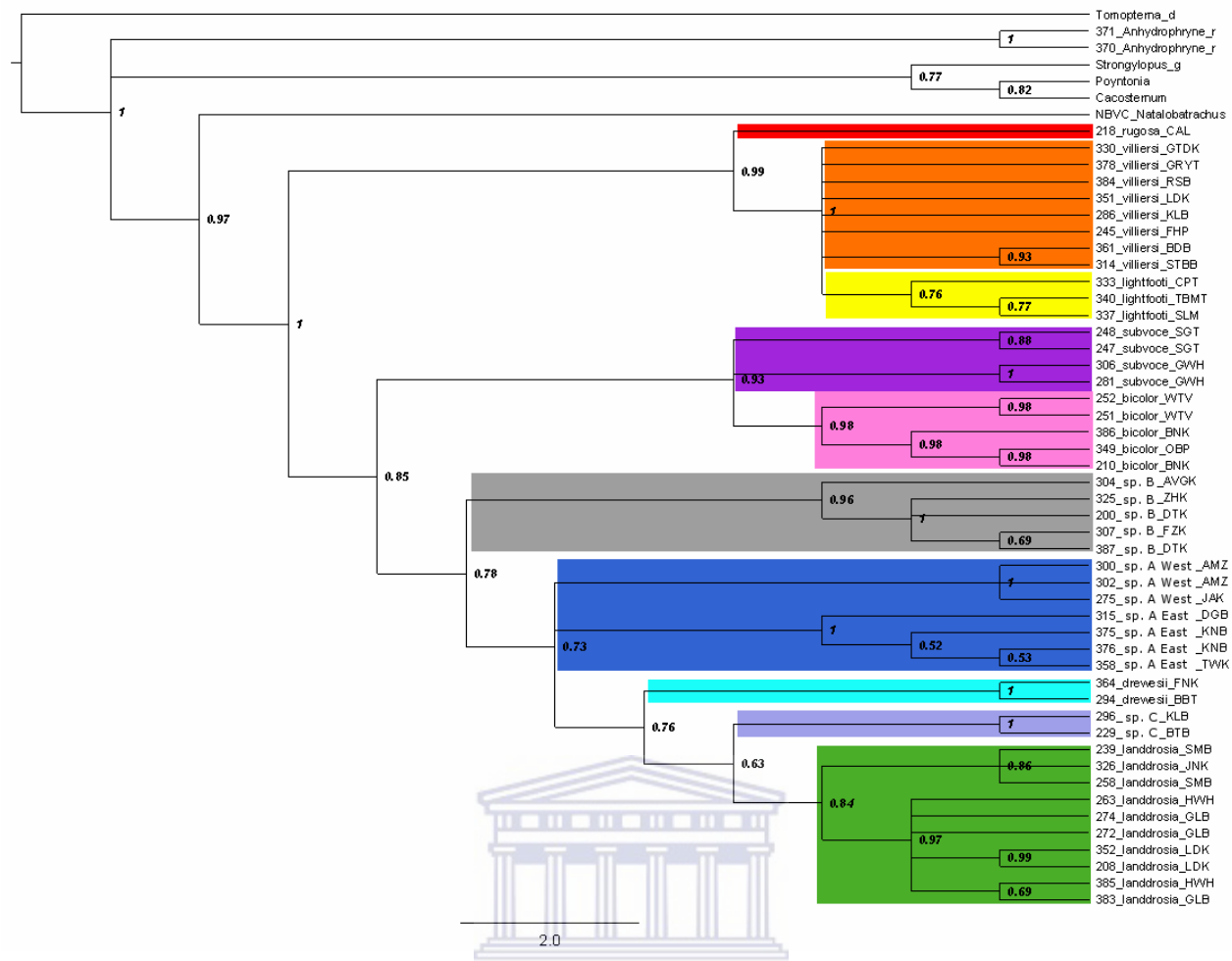

Figure 44. Consensus phylogram derived from direct optimisation analysis of both the 12S and 16S fragments.

Rag-1: Maximum parsimony and direct optimisation methods yielded very poorly resolved trees with numerous basal polytomies within *Arthroleptella* and a number of differences in clade arrangement (Figures 45 and 46). Support values are generally low and Rag-1 did not provide useful phylogenetic signal.

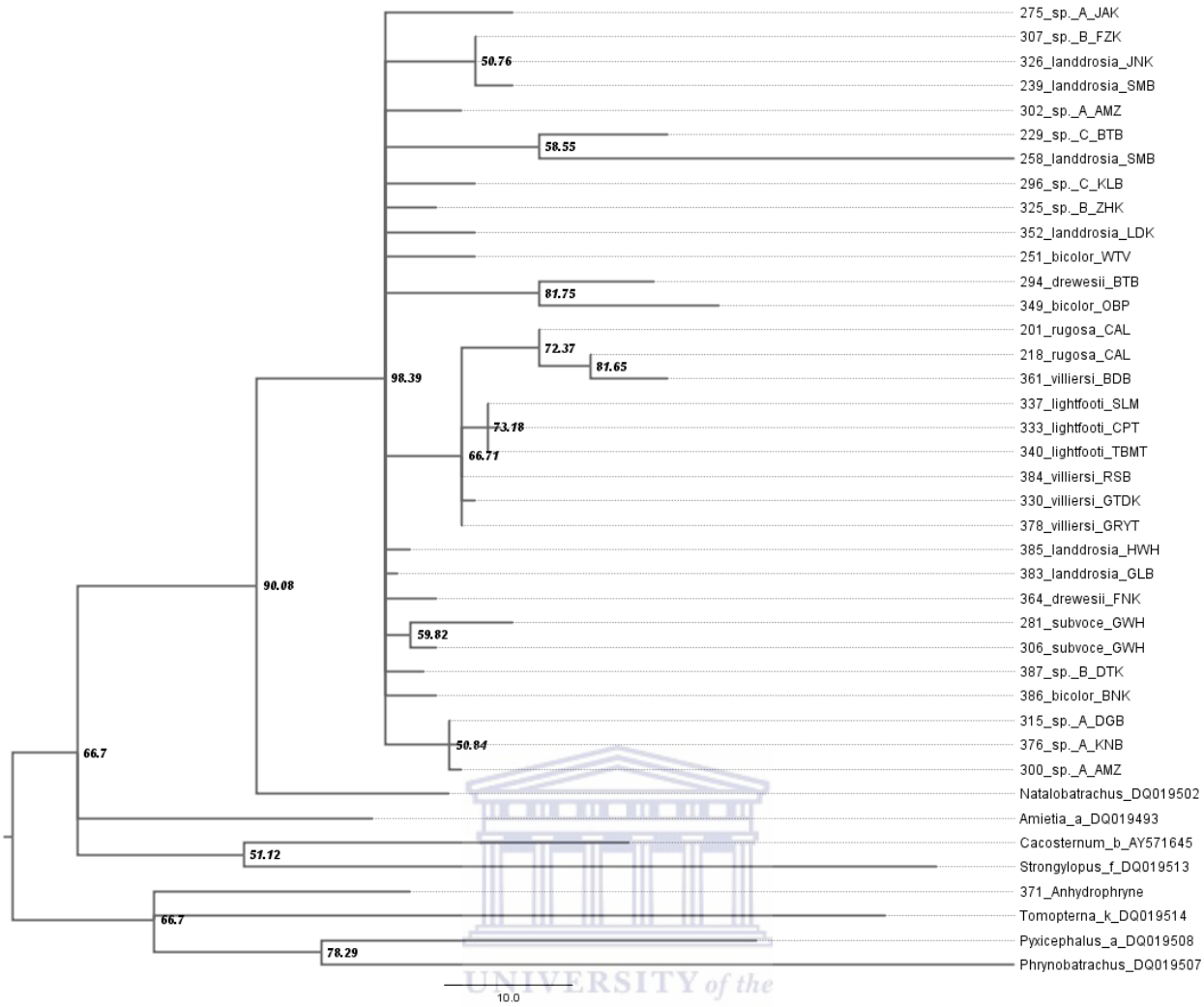

Figure 45. Consensus phylogram derived from parsimony analysis of the Rag-1 fragment displaying multiple polyphyly and poor bootstrap support values on many branches.

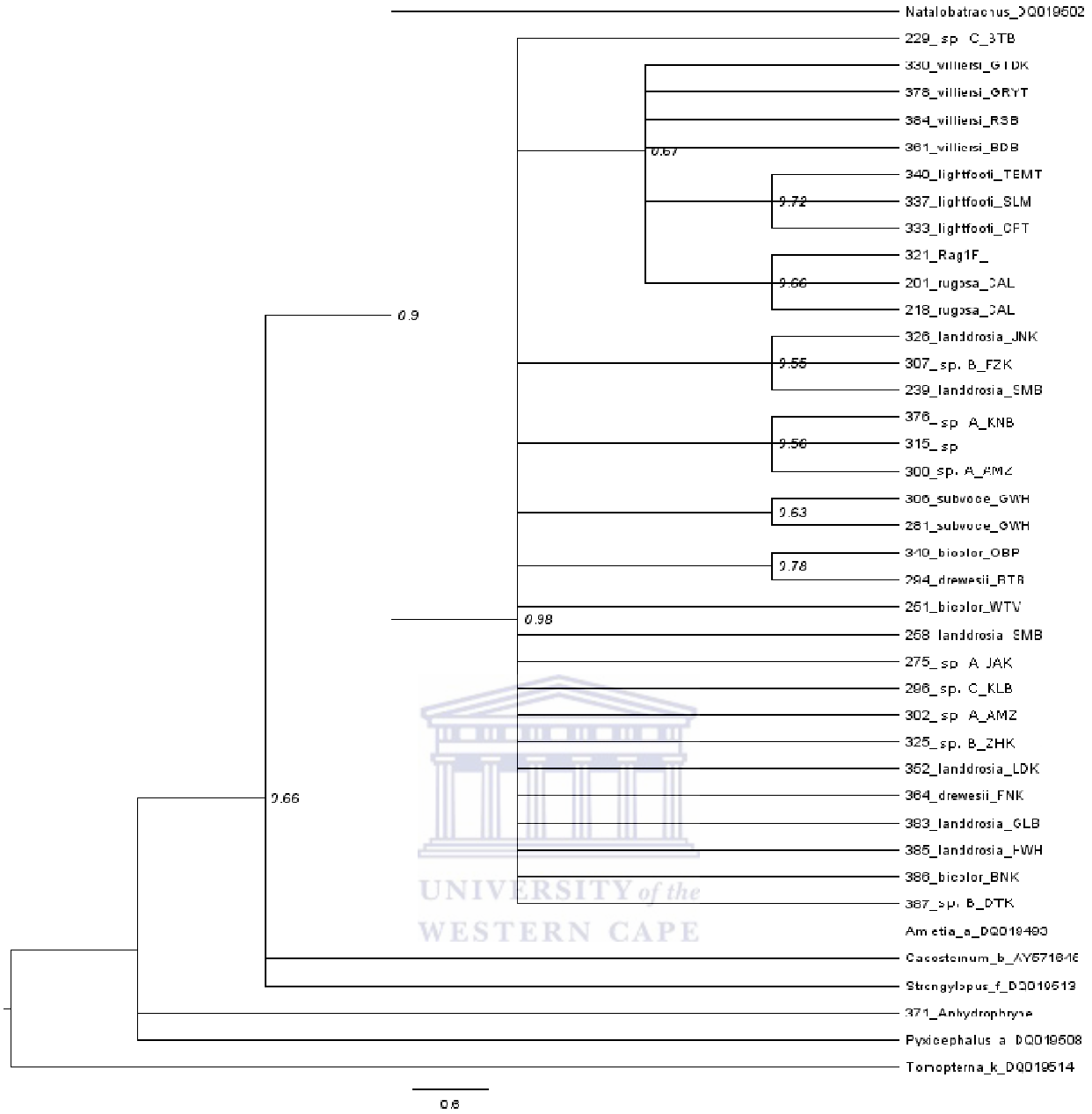

Figure 46. Consensus phylogram derived from direct optimisation analysis of the Rag-1 fragment. Clade colour coding omitted due to lack of resolution.

Mt & Nu: The trees returned by the MP and POY combined mitochondrial and Rag-1 sequences were also well-resolved (Figures 47 and 48) and congruent with the Bayesian combined mitochochondrial and Rag-1 tree (Figure 38).

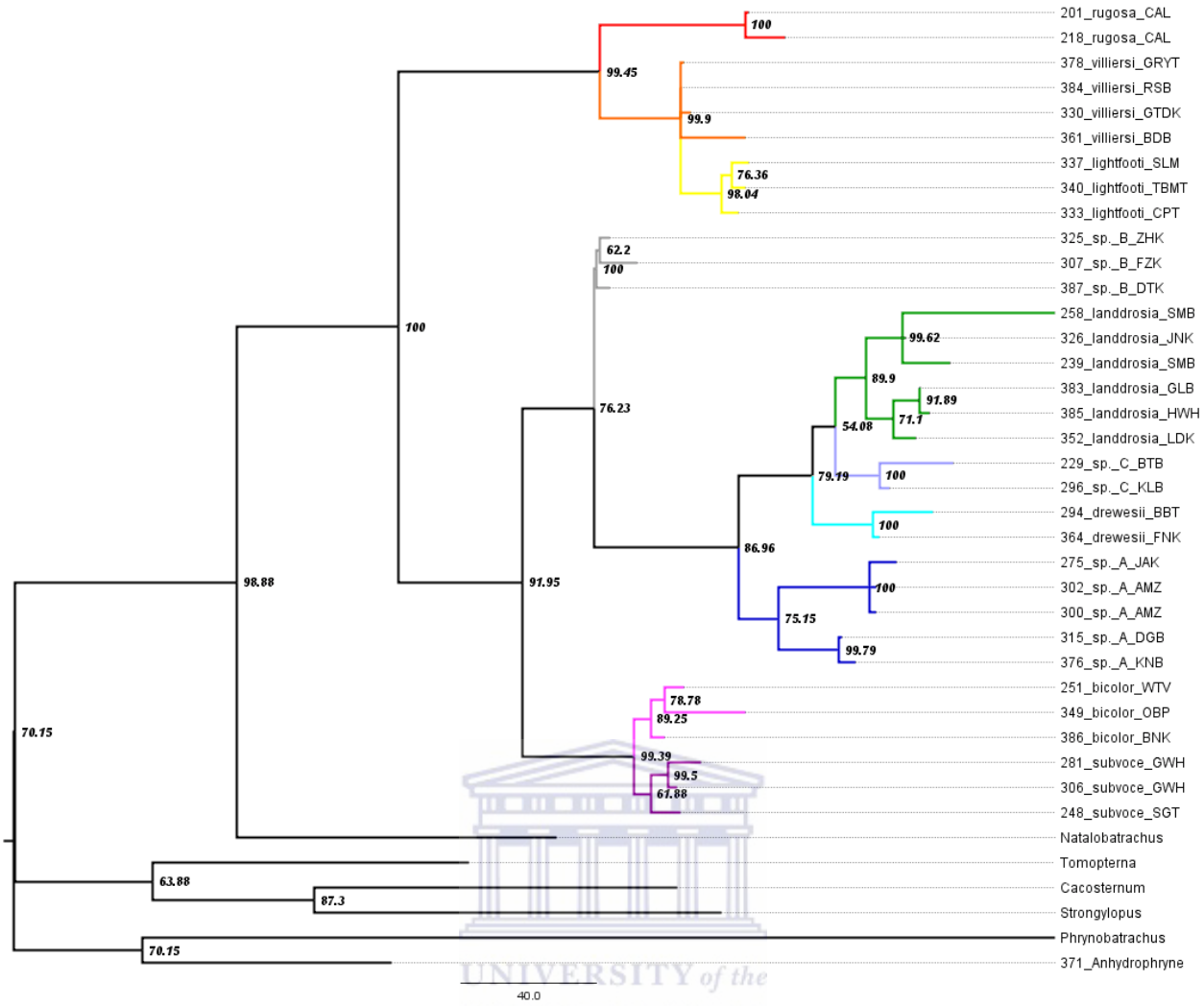

Figure 47. Consensus phylogram derived from parsimony analysis of the combined Rag-1, 12S and 16S fragments.

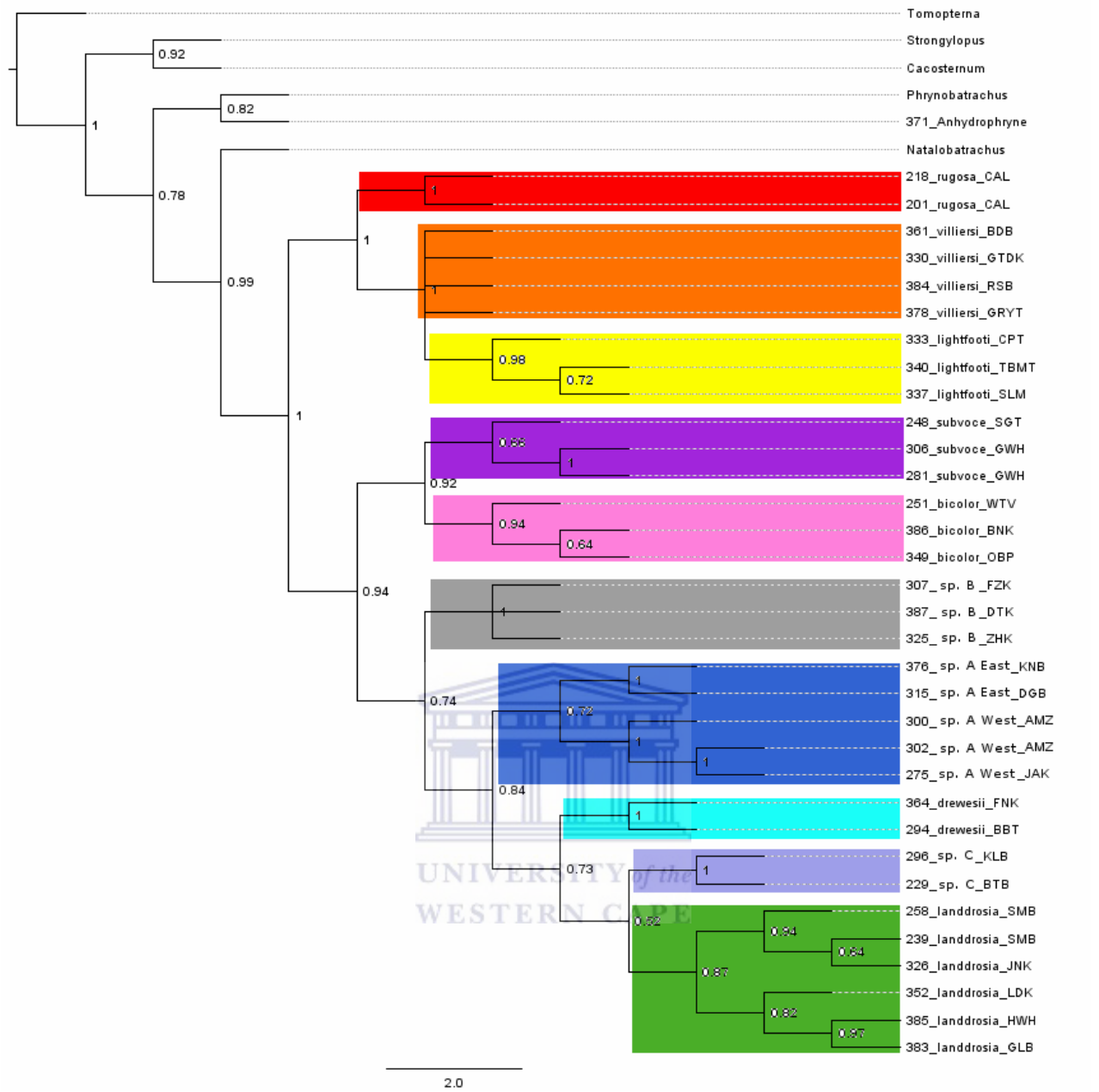

Figure 48. Consensus phylogram derived from direct optimisation analysis of the combined Rag-1, 12S and 16S fragments.

Mitochondrial genetic networks

Median joining networks and reduced median joining networks produced similar topologies and only the latter are presented here.

Table 50. List of abbreviations used in network diagrams.

Species	Abbreviation
Arthroleptella sp. A	sA
Arthroleptella sp. B	sB
Arthroleptella sp. C	sC
Arthroleptella bicolor	BI or BIC
Arthroleptella drewesii	DR or DRE
Arthroleptella landdrosia	LA or LAN
Arthroleptella lightfooti	LI or LIG
Arthroleptella rugosa	RU or RUG
Arthroleptella subvoce	SU or SUB
Arthroleptella villiersi	VI or VIL

12S

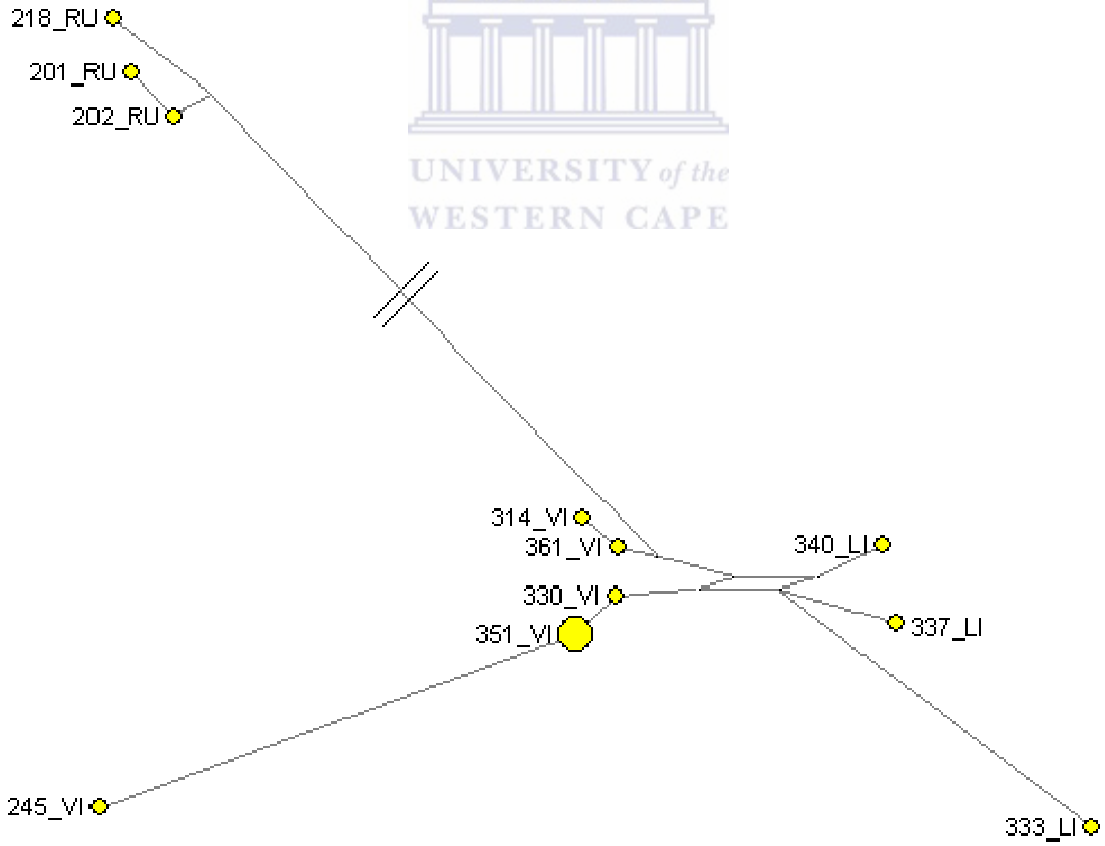

Figure 49. Median joining network diagram for Chirping clade based on 12S gene fragment. The connection to *A. rugosa* has been shortened to fit into the figure.

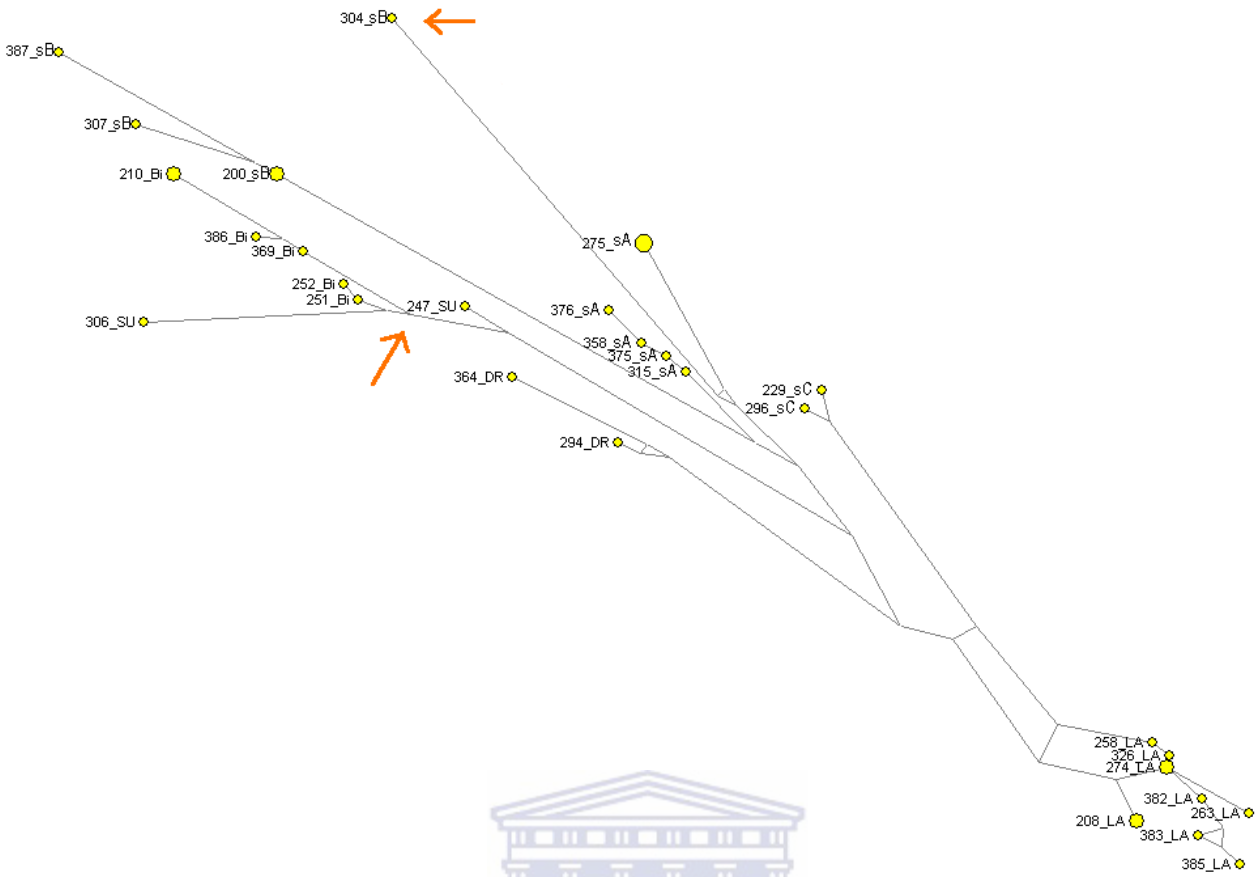

Figure 50. Median joining network diagram for the Clicking clade based on the 12S gene fragment. Note position of specimen 304_sB and paraphyly in the *A. bicolor* and *A. subvoce* clade (marked with arrows).

16S

Figure 51. Median joining network diagram for the Chirping clade based on the 16S gene fragment. Note the linear arrangement of *A. lightfooti* arising from the *A. villiersi* clade.

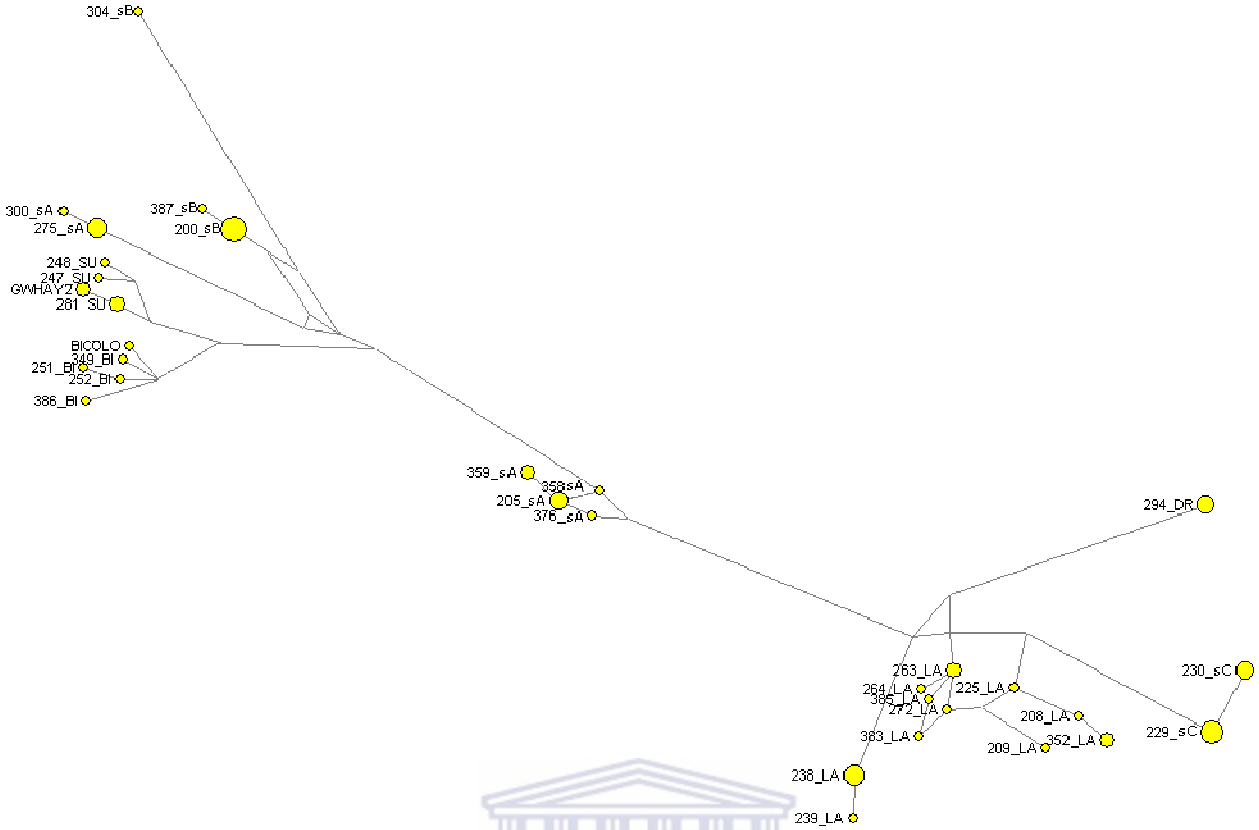

Figure 52. Median joining network diagram for the Clicking clade based on the 16S gene fragment. Note *A. landdrosia* complex with some reticulate relationships.

Mt Combined

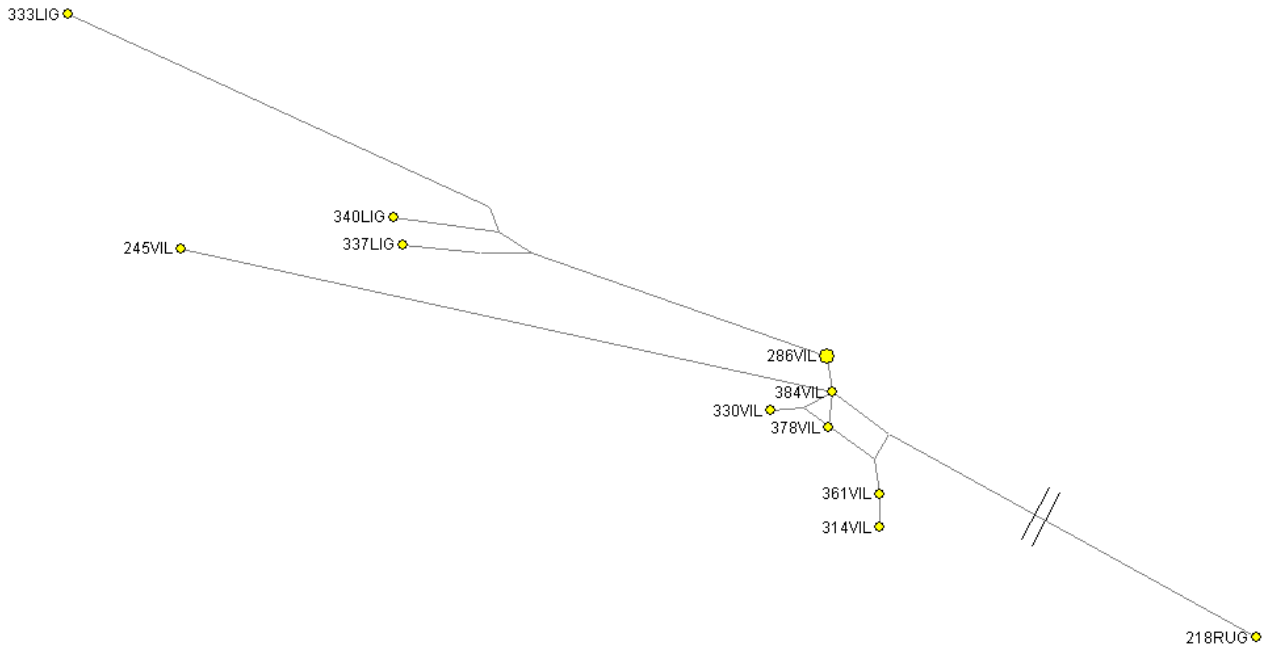

Figure 53. Median joining network diagram for the Chirping clade based on the combined 12S and 16S gene fragments. The connection to *A. rugosa* has been shortened to fit in the image.

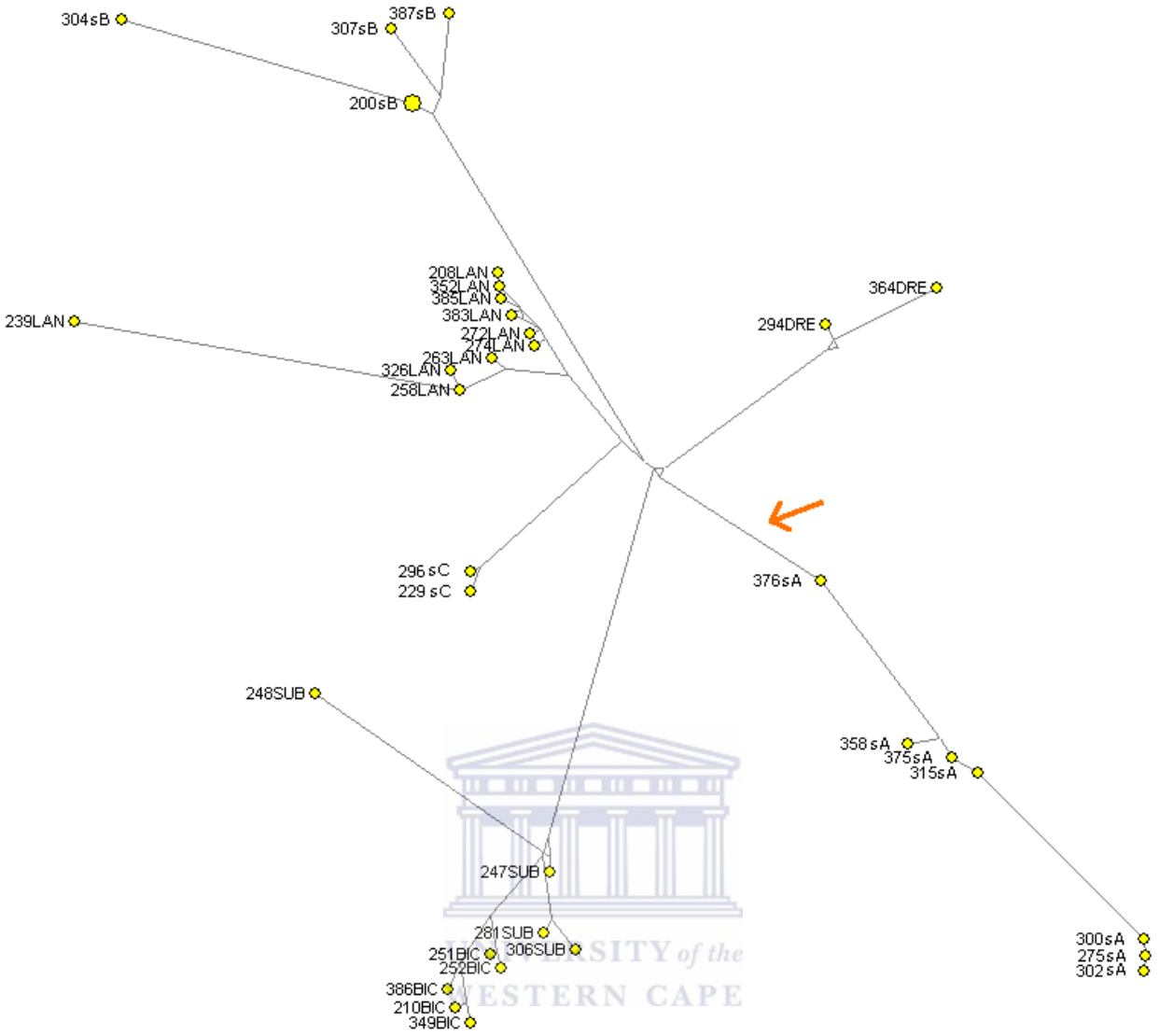

Figure 54. Median joining network diagram for the Clicking clade based on the combined 12S and 16S gene fragments. Note A. sp. Anested in one clade (arrow).

Median joining networks based on the mitochondrial fragments show a number of arrangements of interest:

Chirping clade

The paraphyly of *A. villiersi* and *A. lightfooti* evident in the tree construction methods is also reflected in the network structure. The networks constructed using the 16S and combined 12S and 16S sequences join *A. villiersi* specimens AAT-286 and AAT-351 to the *A. lightfooti* clade (see Figures 51 and Figure 53). Specimen AAT-286 is from the Koëlberg and AAT-351 from the Hottentots-Holland Mountains on the eastern side of the False Bay. The linear arrangement of the *A. lightfooti* is also noteworthy although sample sizes are small (16S = 6, 12S = 3).

Clicking clade

Most populations form non-reticulate clades in the 16S and combined 12S and 16S networks: *A. bicolor, A. subvoce, A. sp.* B and *A. sp.* A East although the last-mentioned clusters in two separate groups in the 16S network (Figure 52). *Arthroleptella landdrosia* is represented as a complex with some reticulate relationships (Figures 37 and 39). A paraphyletic relationship between *A. bicolor* and *A. subvoce* is shown in the 12S network (Figure 50). Within the *A. sp.* B cluster, sample AAT-304 is divergent across all three gene sets.

Estimated timing of speciation events

The BEAST generated timing indicates that the Chirping and click clades coalesced approximately 30 Ma which is close to the coalescence point for *Arthroleptella* and *Natalobatrachus*. The coalescence of *Arthroleptella* species pairs spans the period from approximately 22 to 8 Ma. Coalescence estimates for terminal clades range from 0.1 to 14 Ma (Table 52). As with many other taxonomic groups, the timing of speciation events precedes those derived from Late Pleistocene models of speciation (Bermingham & Moritz 1998). The times generated in the species coalescence data set for the coalescence of *Natalobatrachus* and *Arthroleptella* are somewhat older than those generated in the family phylogeny. This is likely to be the result of using a single nuclear gene (Rag-1) compared to the family phylogeny which included Rhodopsin, Tyrosinase precursor and Rag-2. The faster evolving mitochondrial markers will provide better resolution of more recent cladogenic events although it may lead to a slight over-estimation of coalescence times which may increase towards the terminal branches. The coalescence times for genera obtained from the family-level model should be better estimates than those obtained in the *Arthroleptella* species-level model.

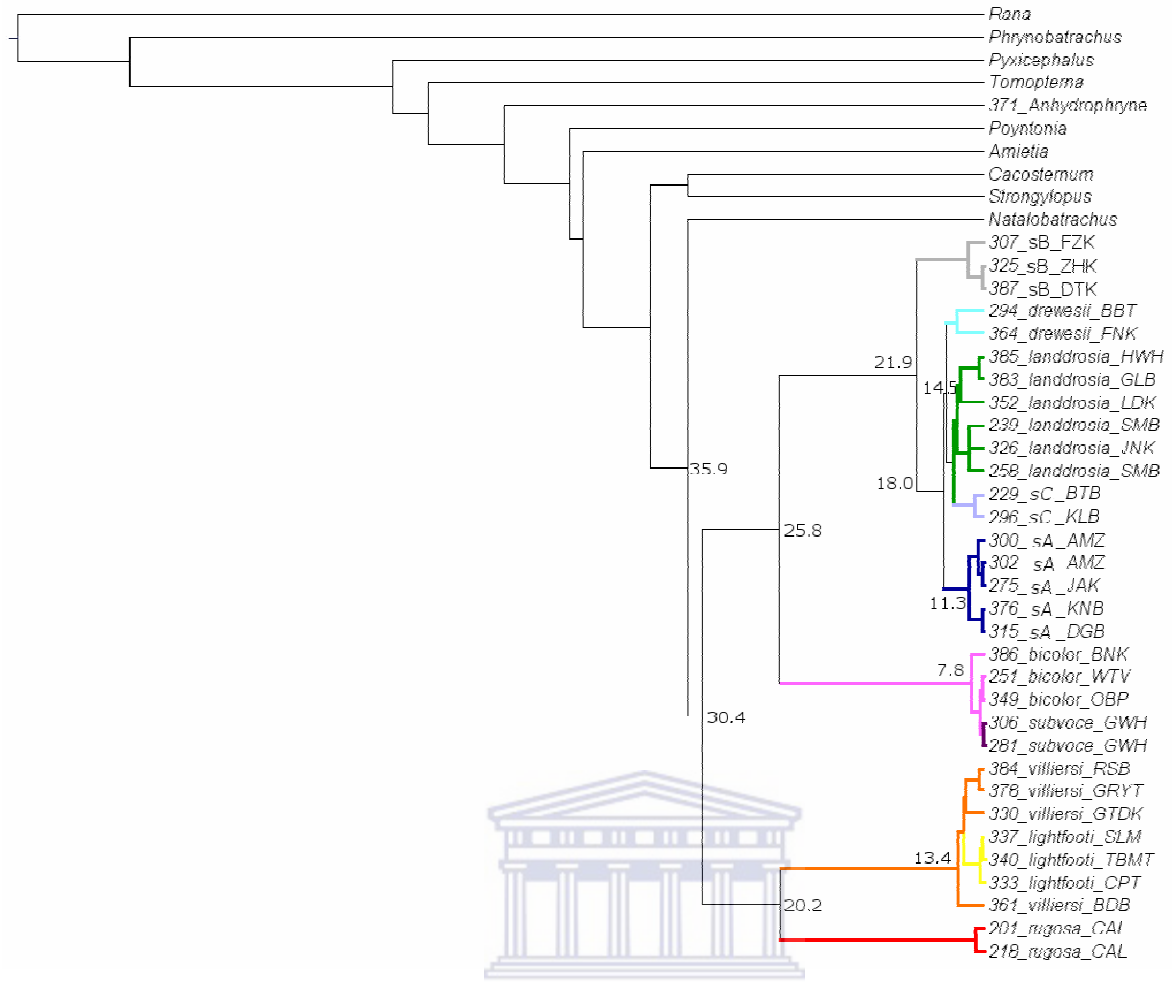

Figure 55. Estimated timing of speciation events from a relaxed-clock Bayesian coalescent method using Rag-1, 12S and 16S gene fragments including an additional age calibration point for the coalescence of the two *A. subvoce* individuals. Errors about these estimates are presented in Table 51 and times for terminal (intra-species) coalescences are presented in Table 52.

Table 51. Standard errors and 95 % highest posterior density (HPD) bounds about the mean time to most recent common ancestor (TMRCA) estimates.

Coalescence time	Mean TMRCA ± S.E. (Ma)	95% lower HPD (Ma)	95% upper HPD (Ma)
35.9	0.6	21.8	48.9
30.4	0.7	15.7	43.4
25.8	1.1	10.6	39.0
21.9	1.1	7.3	34.5
20.2	0.9	5.7	35.3
18	1.1	5.8	30.7
14.5	1.0	3.6	25.9
13.4	0.8	2.1	26.9
11.3	0.7	1.2	22.7
7.8	0.5	0.3	19.4

Table 52. Mean times to most recent common ancestor (TMRCA) within terminal taxa (species) as shown in Figure 55.

Terminal taxon	Mean TMRCA ± S.E. (Ma)	95% lower HPD (Ma)	95% upper HPD (Ma)
A. sp. A East	3.8 ± 0.2	0.01	12.3
A. sp. A West	5.7 ± 0.2	0.1	14.4
A. bicolor	3.5 ± 0.3	0.03	10.6
A. drewesii	5.3 ± 0.3	0.01	15.0
A. sp. B	9.4 ± 0.4	0.2	22.2
A. sp. C	4.3 ± 0.4	0.03	11.6
A. landdrosia	8.3 ± 0.7	0.7	17.6
A. lightfooti	4.8 ± 0.4	0.2	12.4
A. rugosa	4.4 ± 0.2	0.002	15.0
A. subvoce	0.1 ± 0.4	0.0007	0.4
A. villiersi	13.4 ± 0.9	2.1	26.9

Evolutionary rate was constant throughout the relaxed-clock Bayesian coalescent tree with a slight increase in the terminals of *A. subvoce*.

The shape of the estimated parameter distributions were normal to slightly log-normal, with the exception of times to the most recent common ancestor within species where the was a strong skew towards younger dates as would be expected as speciation times within the genus are young compared to the evolution of the entire family. The addition of an age calibration point (gamma

distribution from 0 to 500 000 years with k = 0.5 and θ = 0.2) for the coalescence of two *A. subvoce* individuals that are likely to have shared a recent common ancestor based on close geographical proximity, decreased the age estimation for terminal coalescences but had a negligible effect on internal nodes.

3.4.5 Phylogeography

Mantel tests

The results of the application of Mantel tests to the 16S mitochondrial sequences is displayed graphically below for the full data set, Clicking clade, Chirping clade and Landdrosia clade.

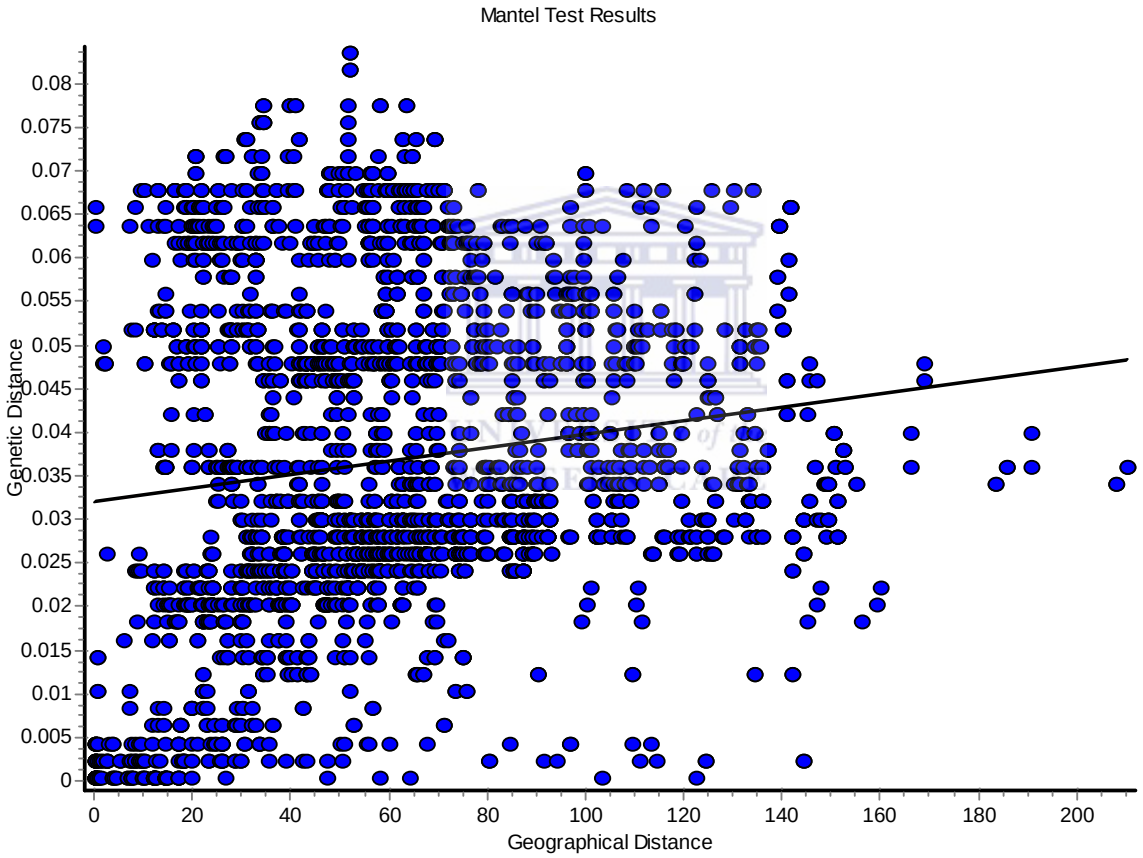

Figure 56. Scatter plot of genetic vs. geographic distance (km) for all 16S mitochondrial sequences from Mantel test; R- = 0.15, p ≥ observed < 0.001.

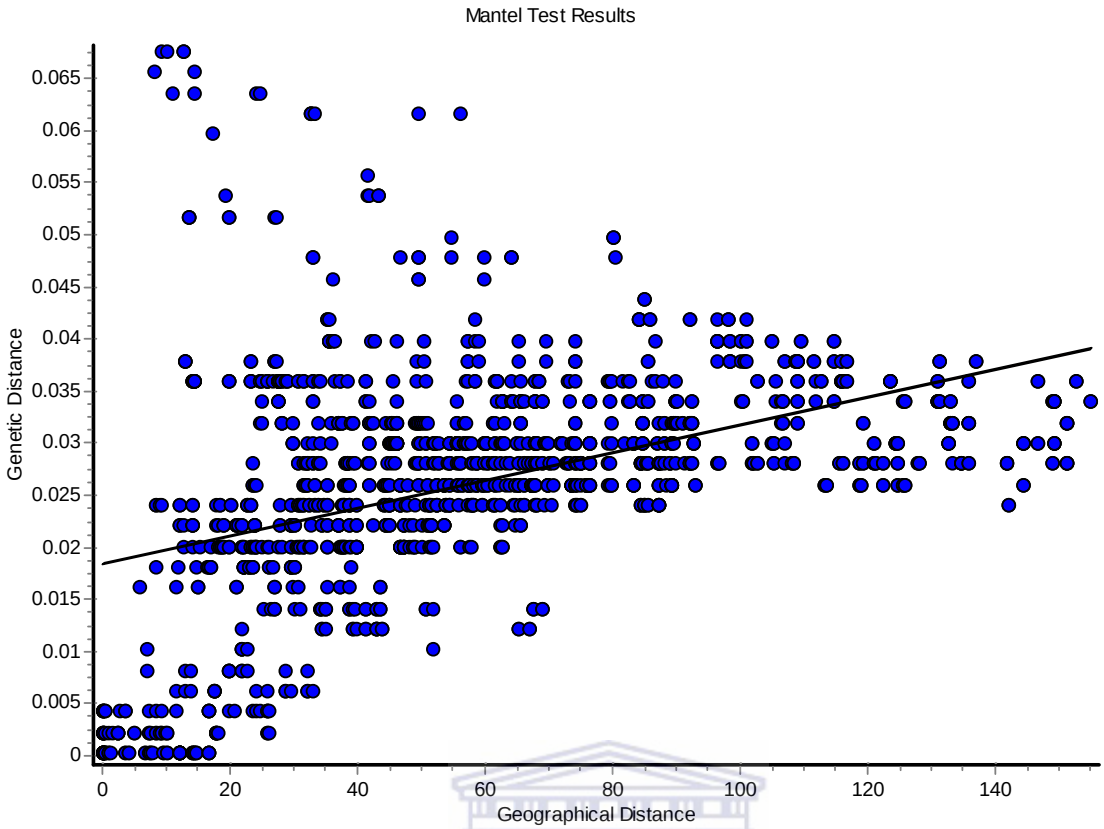

Figure 57. Scatter plot of genetic vs. geographic distances (km) for 16S mitochondrial sequences for the Clicking clade from Mantel test; R = 0.43, p ≥ observed < 0.001.

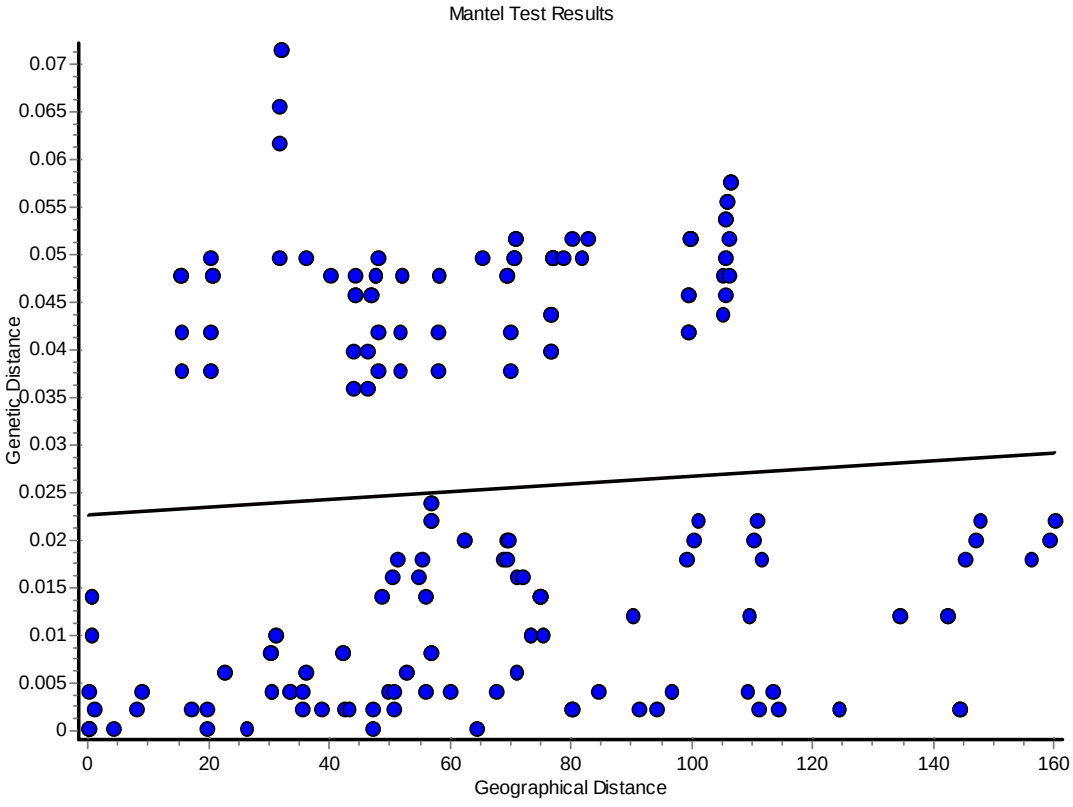

Figure 58. Scatter plot of genetic vs. geographic distances (km) for 16S mitochondrial sequences for the Chirping clade from Mantel test; R = 0.07, p ≥ observed = 0.25.

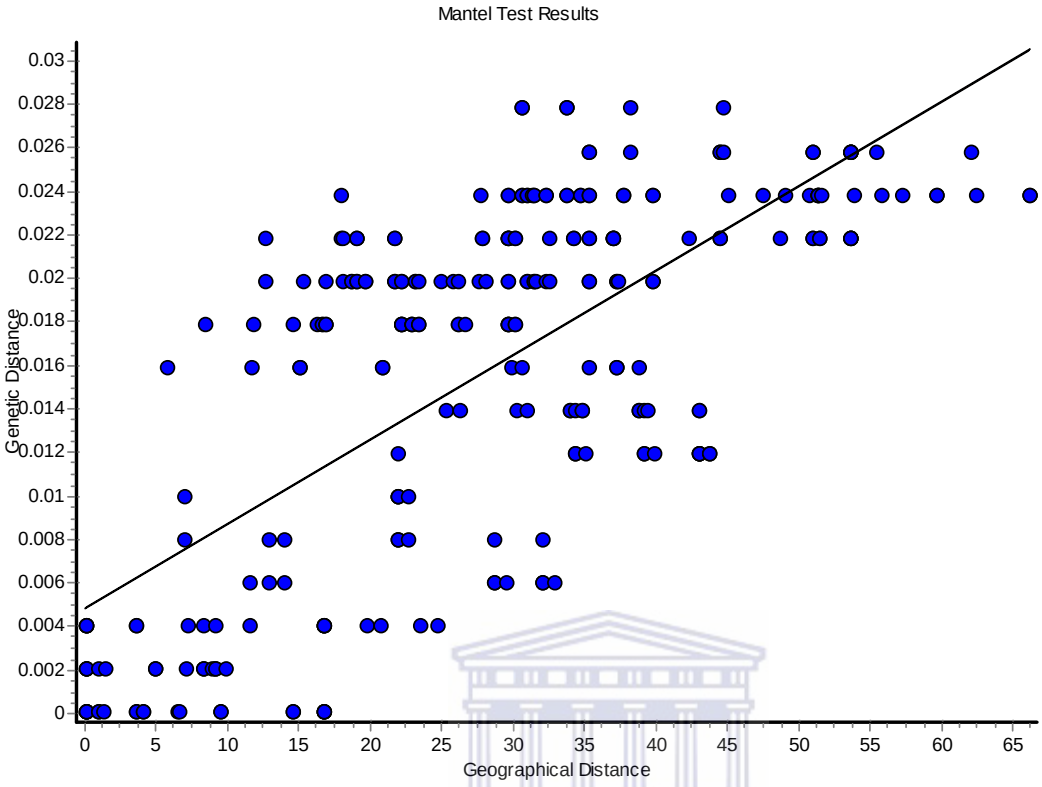

Figure 59. Scatter plot of genetic vs. geographic distances (km) for 16S mitochondrial sequences for the Landdrosia clade from Mantel test; r-value 0.70, p-value ≥ observed < 0.001.

The Mantel tests indicate a weak positive relationship between spatial and genetic distance for all groups (Figures 43 to 46) except for the Landdrosia clade which shows a moderate positive correlation. All these relationships are significant at alpha = 0.05 except for those in the Chirping clade.

Spatial autocorrelation

The spatial autocorrelation diagrams for the entire genus, the Clicking clade and the Chirping clade are shown below. In these diagrams Ay on the y-axis is average genetic distance between pairs of individuals that fall within distance class y (Miller 2005). Spatial autocorrelation was statistically significant for the Clicking clade and the Landdrosia clade ($p < 0.001$) but was not significant for the genus as a whole ($p = 0.34$) and marginally non-significant ($p = 0.055$) in the Chirping clade.

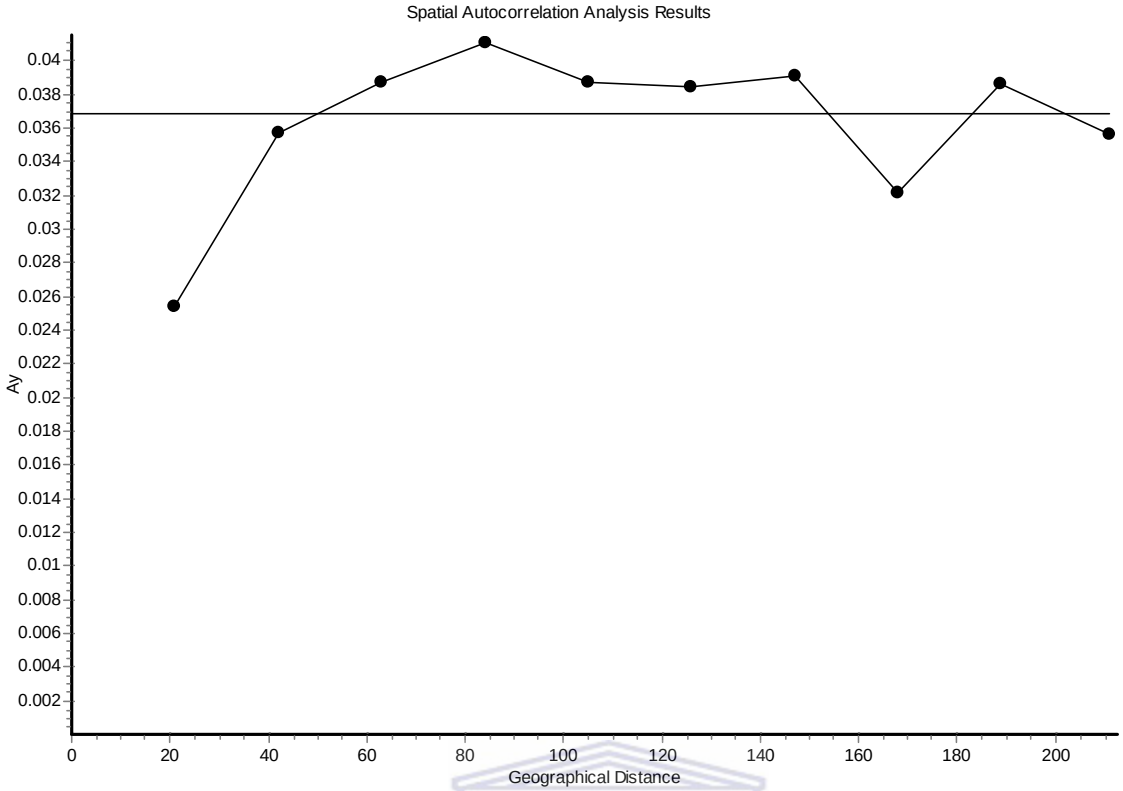

Figure 60. Spatial autocorrelation diagram for all *Arthroleptella* sampled showing a general pattern of increasing genetic variation with geographic distance (in km) until 85 km.

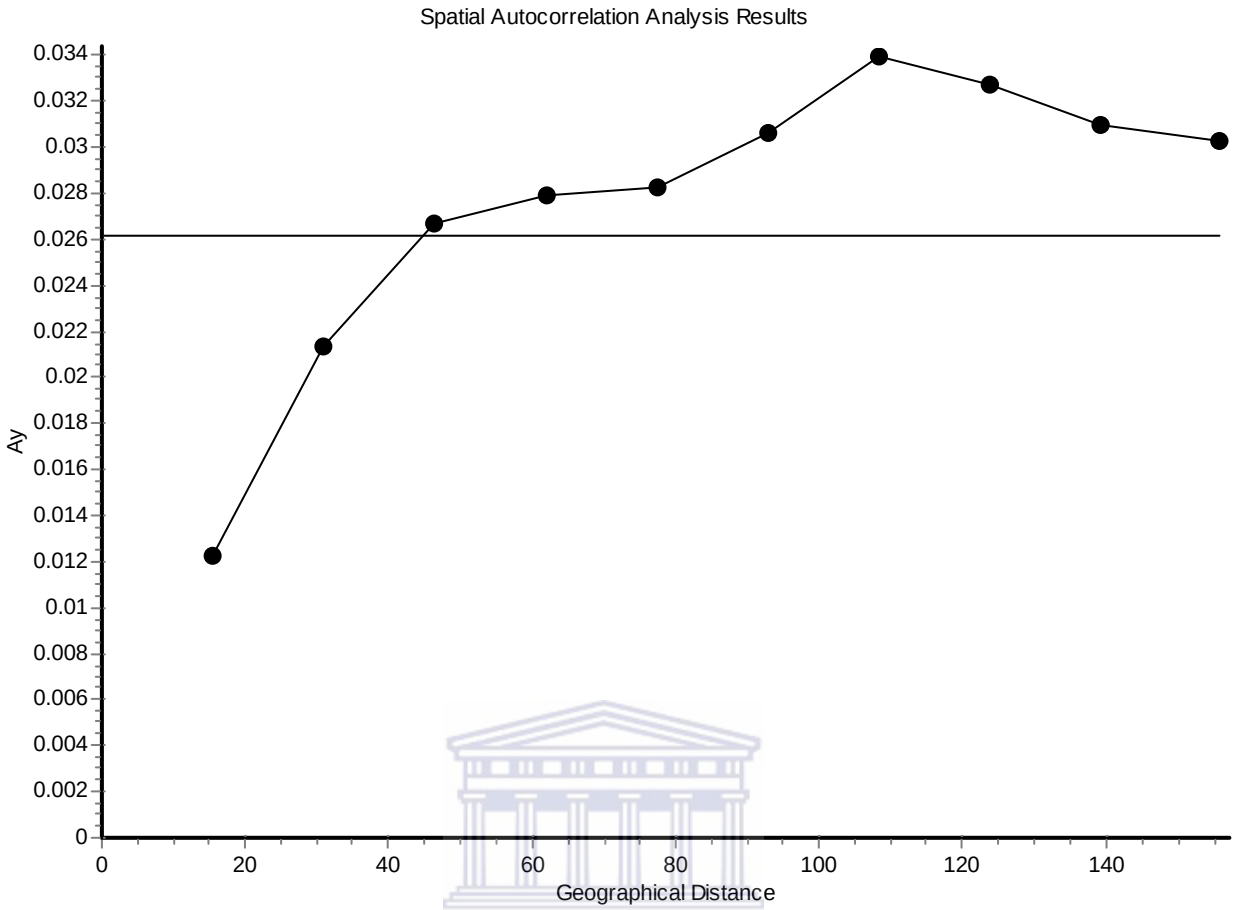

Figure 61. Spatial autocorrelation diagram for the Clicking clade only showing a general pattern of increasing genetic variation with geographic distance (in km) until 110 km.

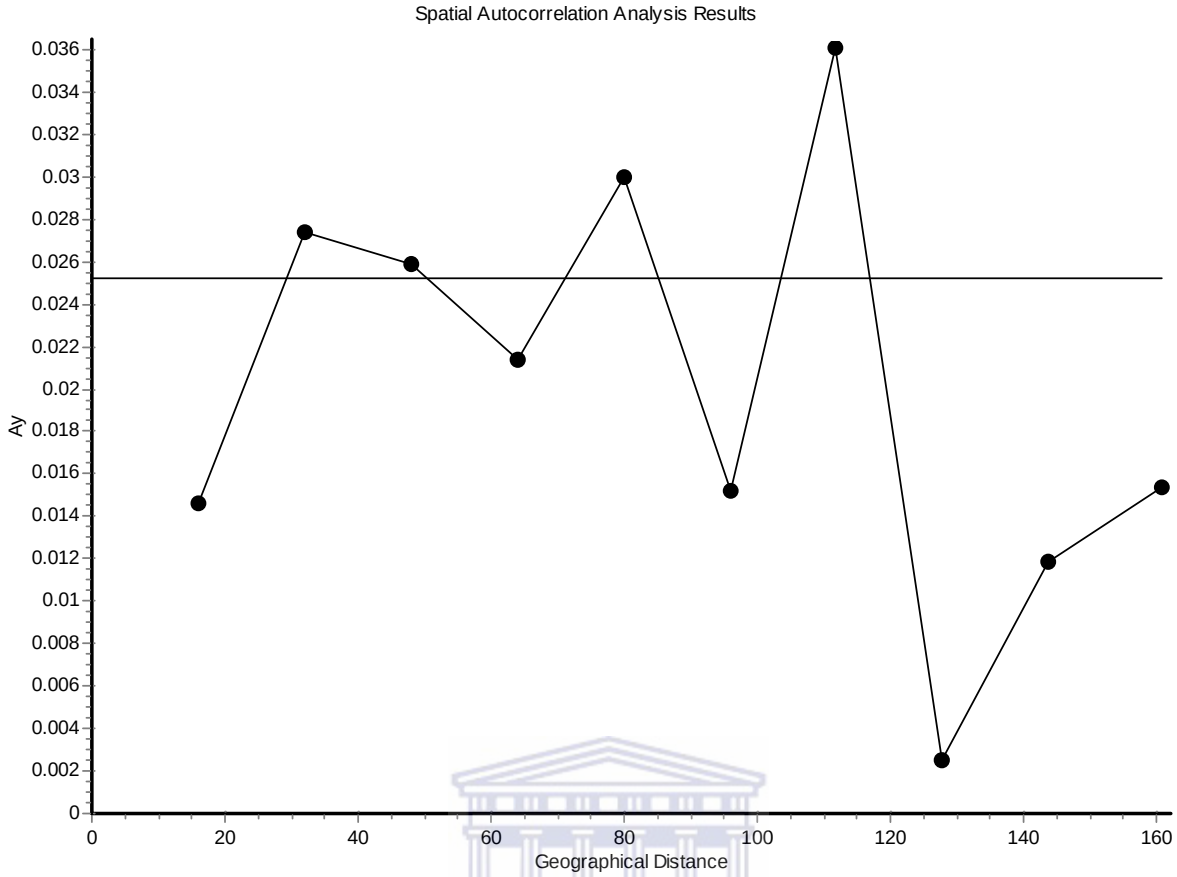

Figure 62. Spatial autocorrelation diagram for the Chirping clade only showing an unclear relationship between genetic variation and geographic distance (in km).

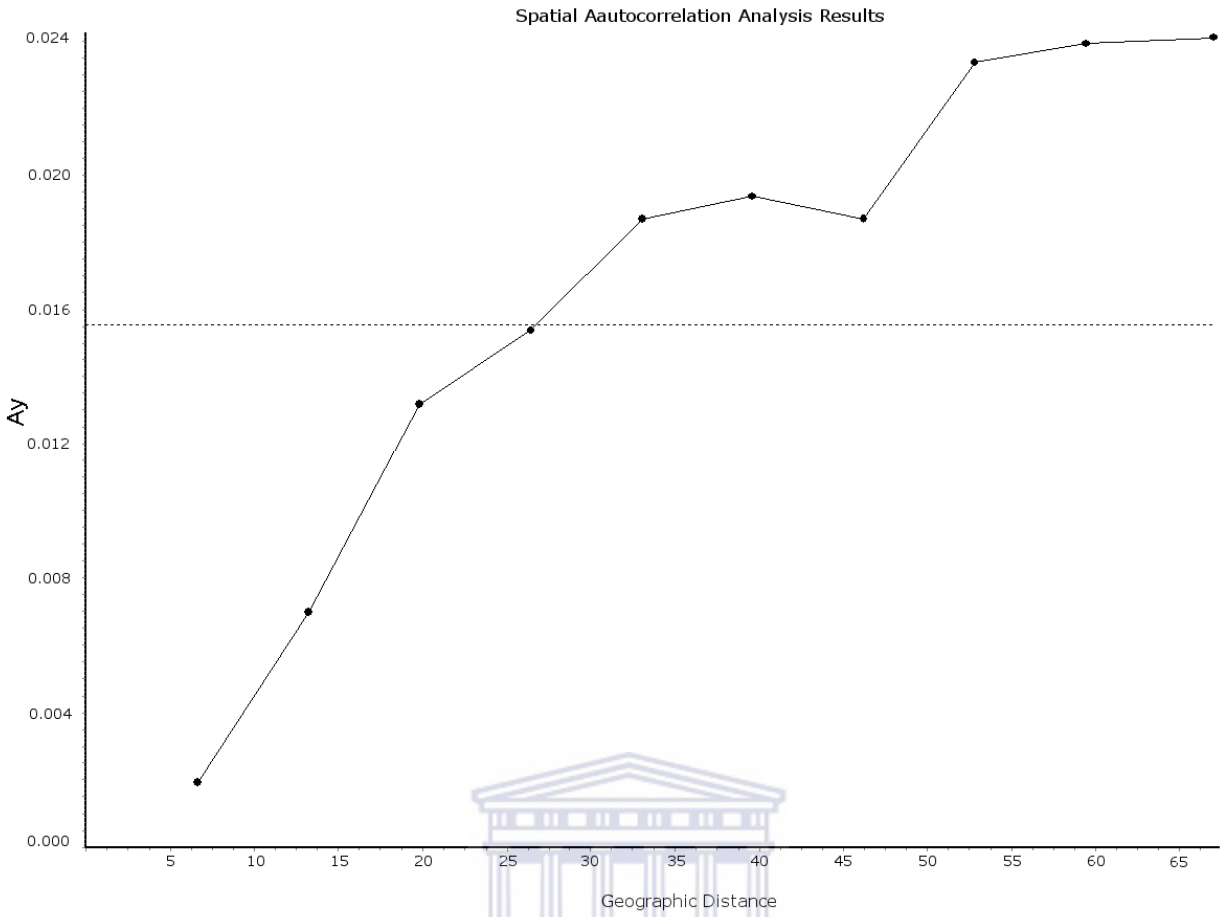

Figure 63. Spatial autocorrelation diagram for the Landdrosia clade only in which a clear positive relationship between genetic variation and geographic distance (in km) is displayed across the entire range.

The spatial autocorrelation diagrams show positive spatial autocorrelation of genetic distance over geographic distances less than 85 km for the genus as a whole (Figure 60) and less than 110 km for the Clicking clade (Figure 61). At greater distances the relationship weakens and becomes negative. There is spatial autocorrelation over the entire geographic range of the Landdrosia clade (Figure 61). The relationship in the Chirping clade is less clear indicating a lack of isolation by distance for distances above 15 km and less than 130 km (Figure 62).

Monmonier analyses

Monmonier algorithm distance analyses for group sizes of one to eleven were generated and are displayed in Figure 64 below.

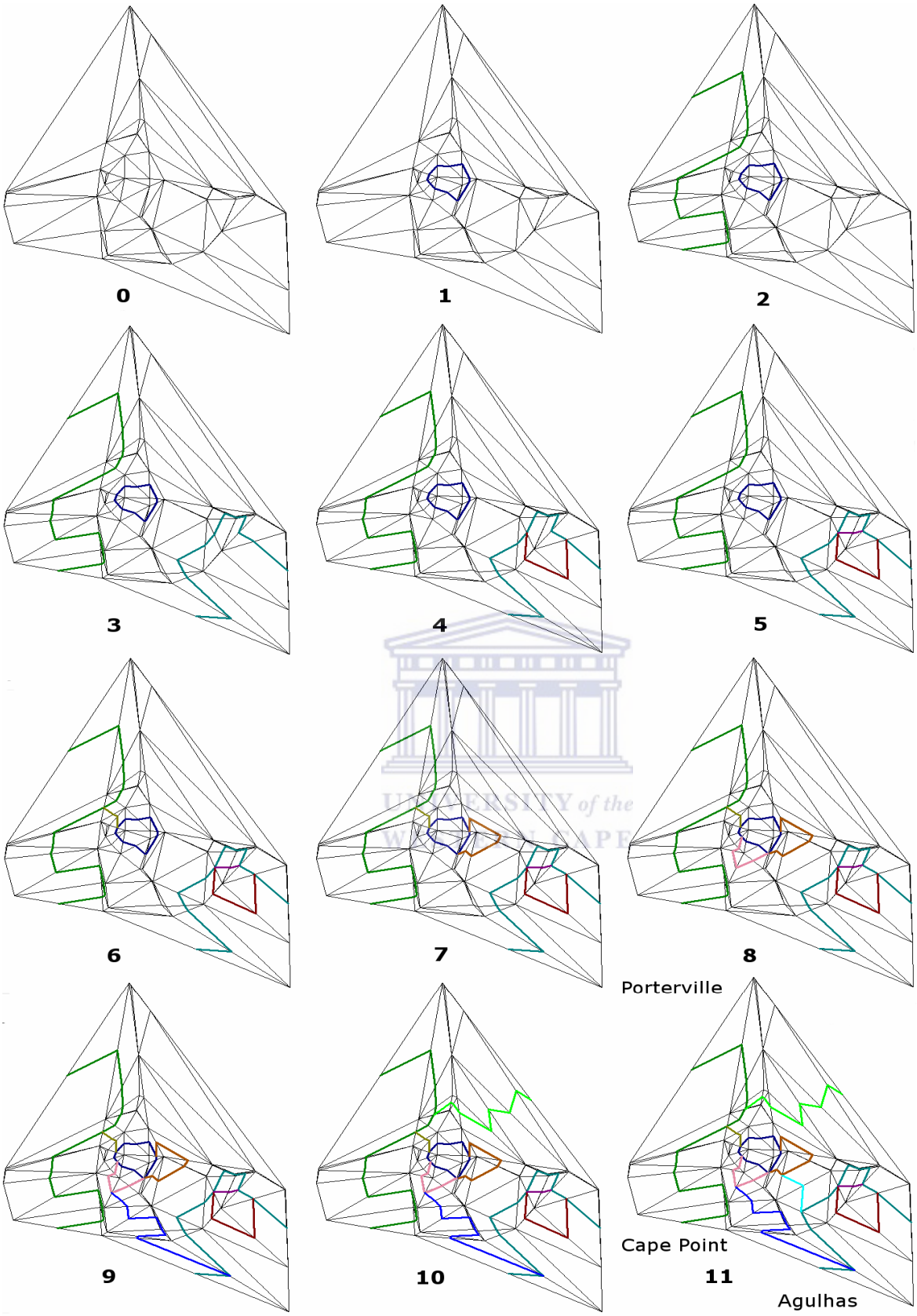

Figure 64. The stepwise location of barriers to gene flow as indicated by the application of the Monmonier algorithm over an increasing number of groups. Each step is represented by a different colour.

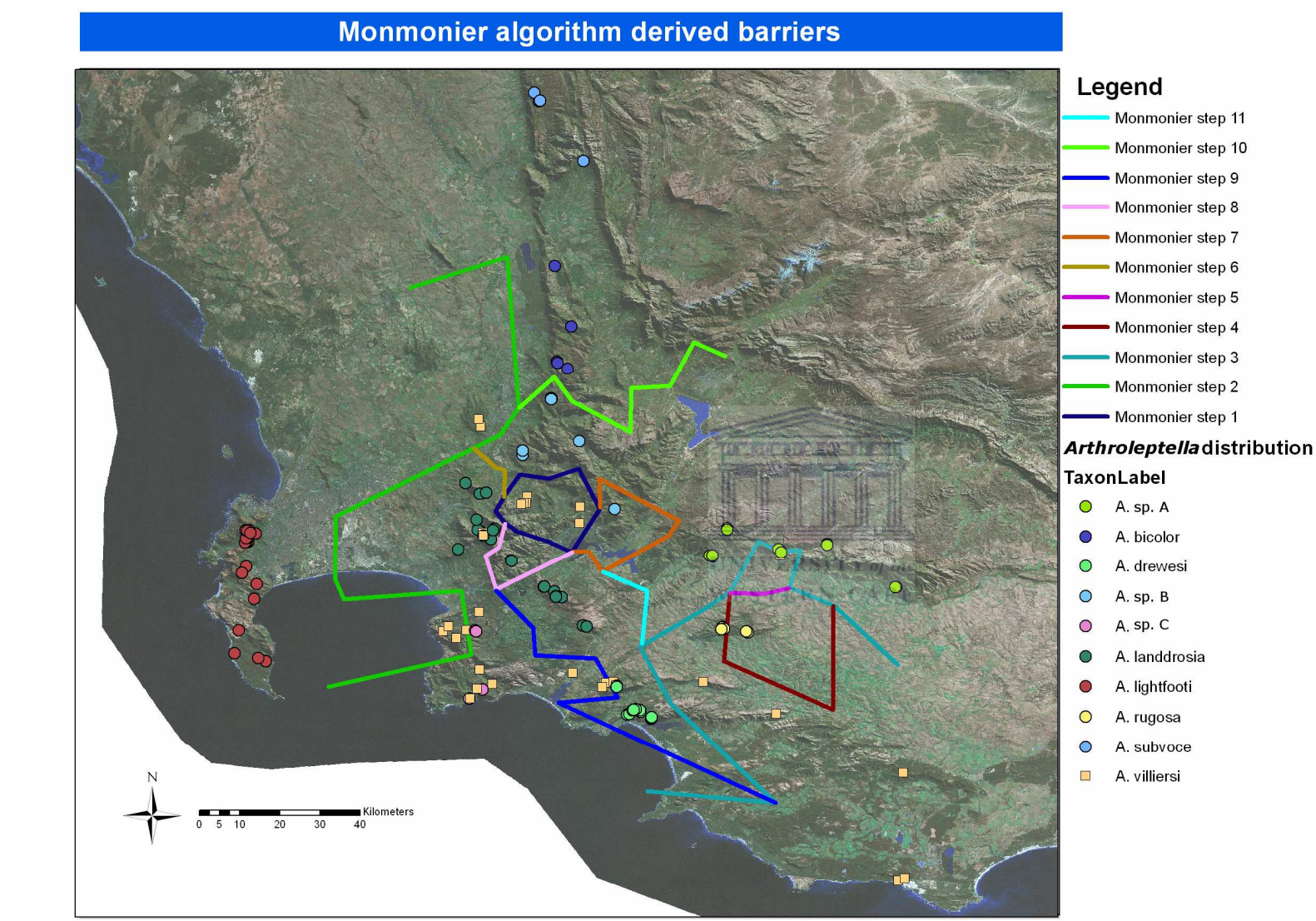

Figure 65. Location in geographical space of the barriers identified by the application of the Monmonier algorithm.

This procedure identified barriers that correspond to the geographic and taxonomic features represented in Table 53.

Table 53. Barriers derived from Monmonier analysis with interpretation of taxonomic implications. Barrier numbers correspond with steps in Figure 64 and Figure 65.

Barrier number	Geographic feature	Taxonomic boundary interpretation
1	Du Toitskloof Mountains.	*A. sp.* B isolated from all other species.
2	Lowlands of Cape Flats and West Coast isolating Peninsula population	*A. lightfooti* isolated from all other species.
3	South eastern lowlands	Separates the easternmost *A. villiersi* from the remaining *A. villiersi*.
4	Klein Swartberg inselberg near Caledon and low-lying hills to the Riviersonderend Mountains	Includes distribution of *A. rugosa* plus some unsuitable habitat between the Klein Swartberg and the Riviersonderend Mountains.
5	Klein Swartberg inselberg near Caledon	Distribution of *A. rugosa on* the Klein Swartberg inselberg. (See discussion of western boundary in text.)
6	Lowlands south of Paarl to the Du Toitskloof Mountains	Lowlands isolating the Paarl Mountain inselberg.
7	Mountain range NW of Villiersdorp	Location of a very isolated and unique population of *A. sp*. A.
8	Southern bounding valleys of the Groot Drakenstein	Separates northern *A. villiersi* from southern *A. villiersi.*
9	Kogelberg to Hermanus mountainous coastal areas	Separates *A. drewesii* and Kogelberg Clicking clade from *A. landdrosia.*
10	Berg and Breede River valleys	Separates *A. bicolor* and *A. subvoce* from all other species.
11	Lowlands of western Overberg	Separates *A. landdrosia* from Riviersonderend populations of the Clicking clade.

The Monmonier algorithm indicated a barrier through the *A. rugosa* distribution where there is no reason to expect a major discontinuity in the distribution of the species. The cause of the position of this barrier is a 16S sequences from the western part of the range was not available so the algorithm was not aware of the existence of a sample on the other side of the identified barrier.

Genetic landscapes

The results of the interpolation of genetic differentiation across the landscape are depicted in Figures 53 to 55.

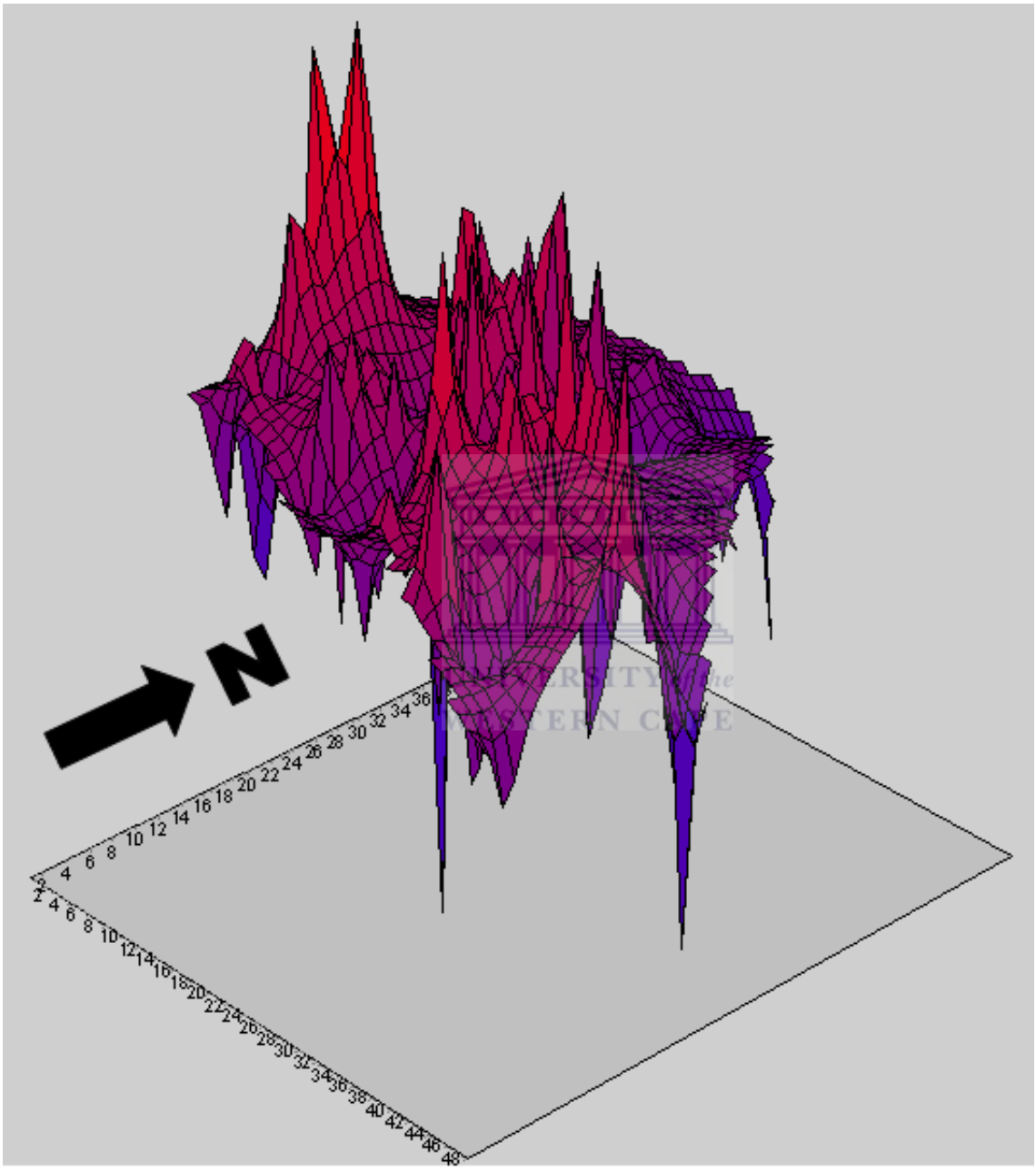

Figure 66. Genetic landscape for entire genus as interpolated from AIS. The arrow indicates north with the highest peaks in genetic diversity in the west.

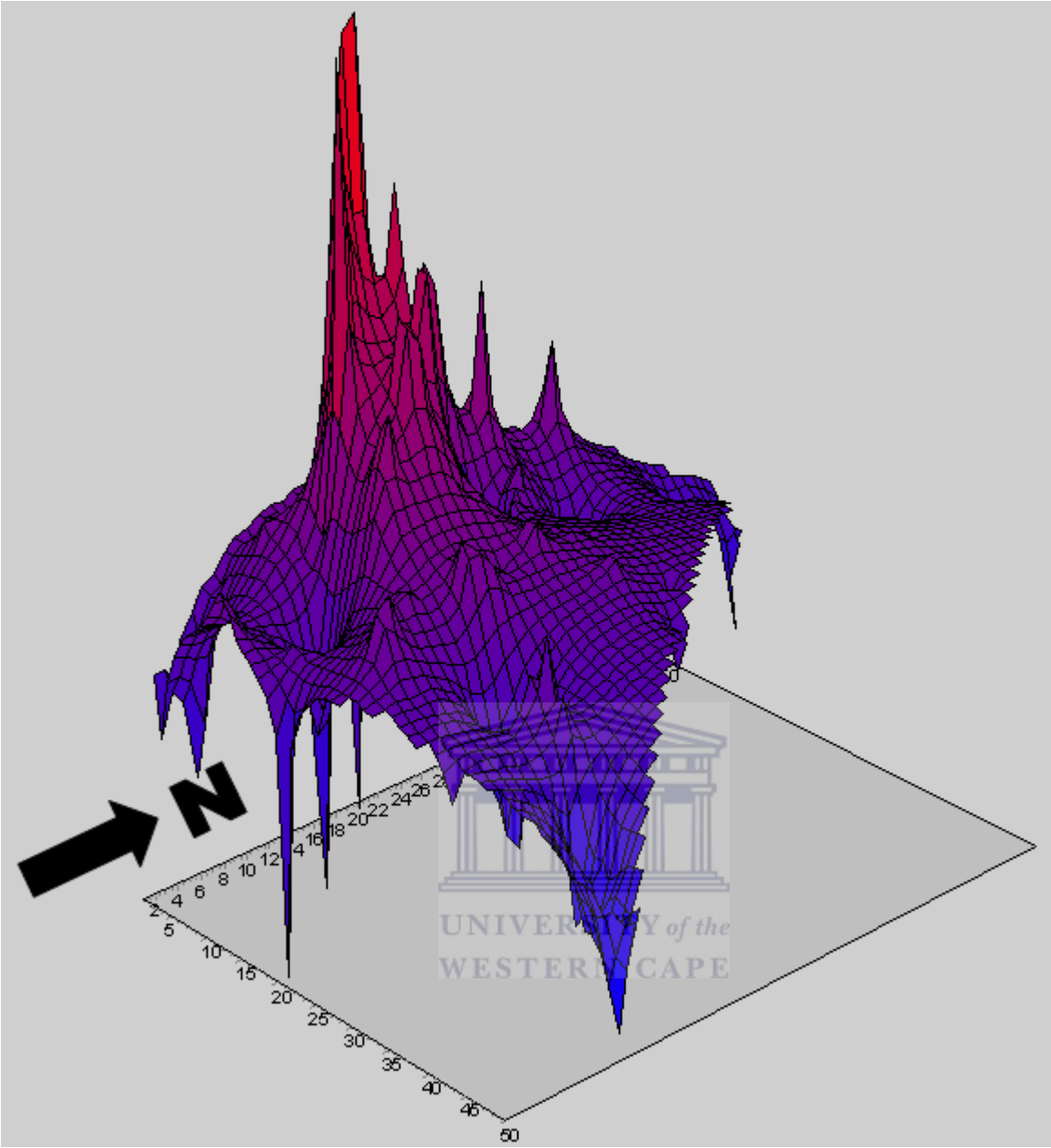

Figure 67. Genetic landscape for Clicking clade as interpolated from AIS showing a clear peak in genetic diversity in the west.

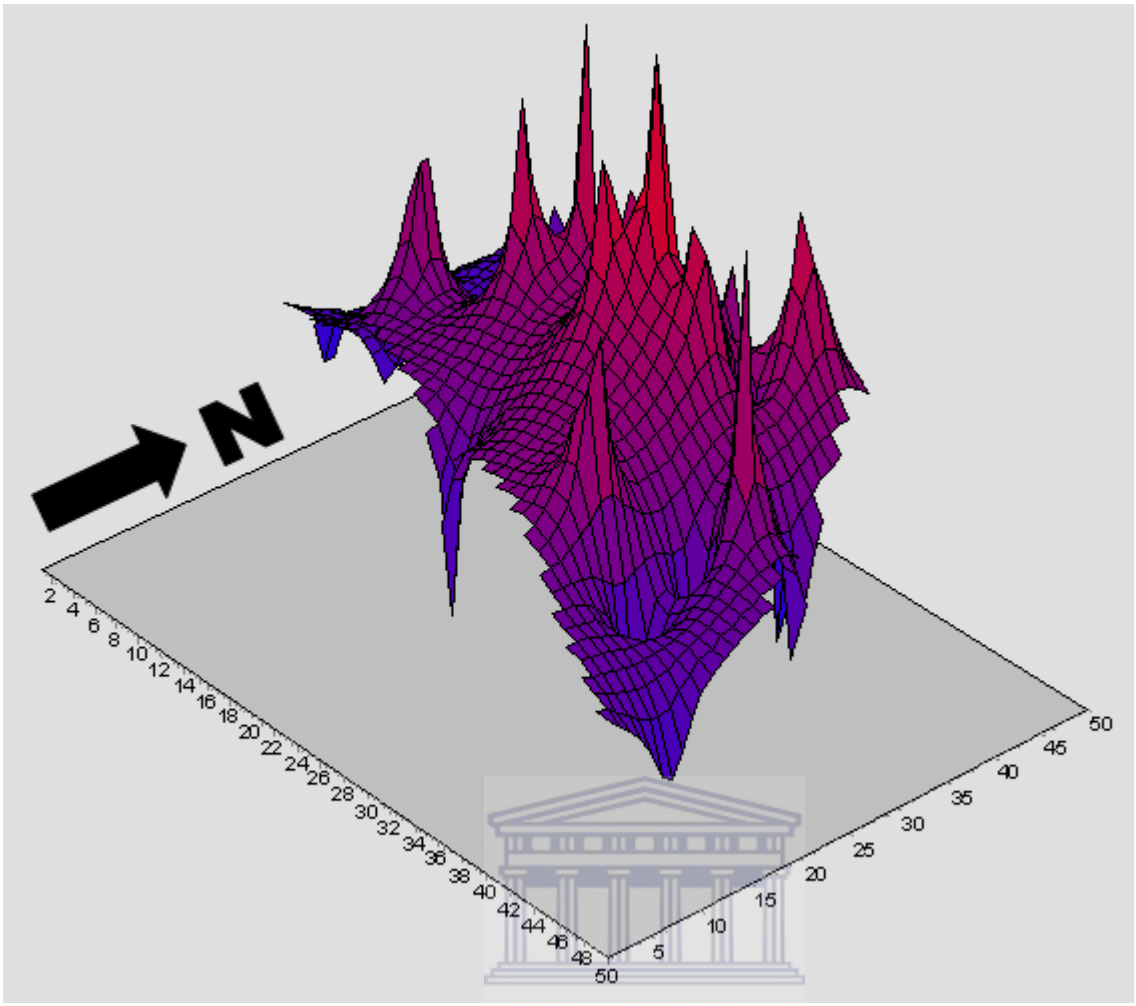

Figure 68. Genetic landscape for Chirping clade as interpolated from AIS.

The genetic landscape diagrams indicate a northward decline in genetic diversity for the genus as a whole. The Clicking clade displays a marked western peak in genetic diversity whereas the Chirping clade shows a more uniform spatial distribution with a slight tail off to the east.

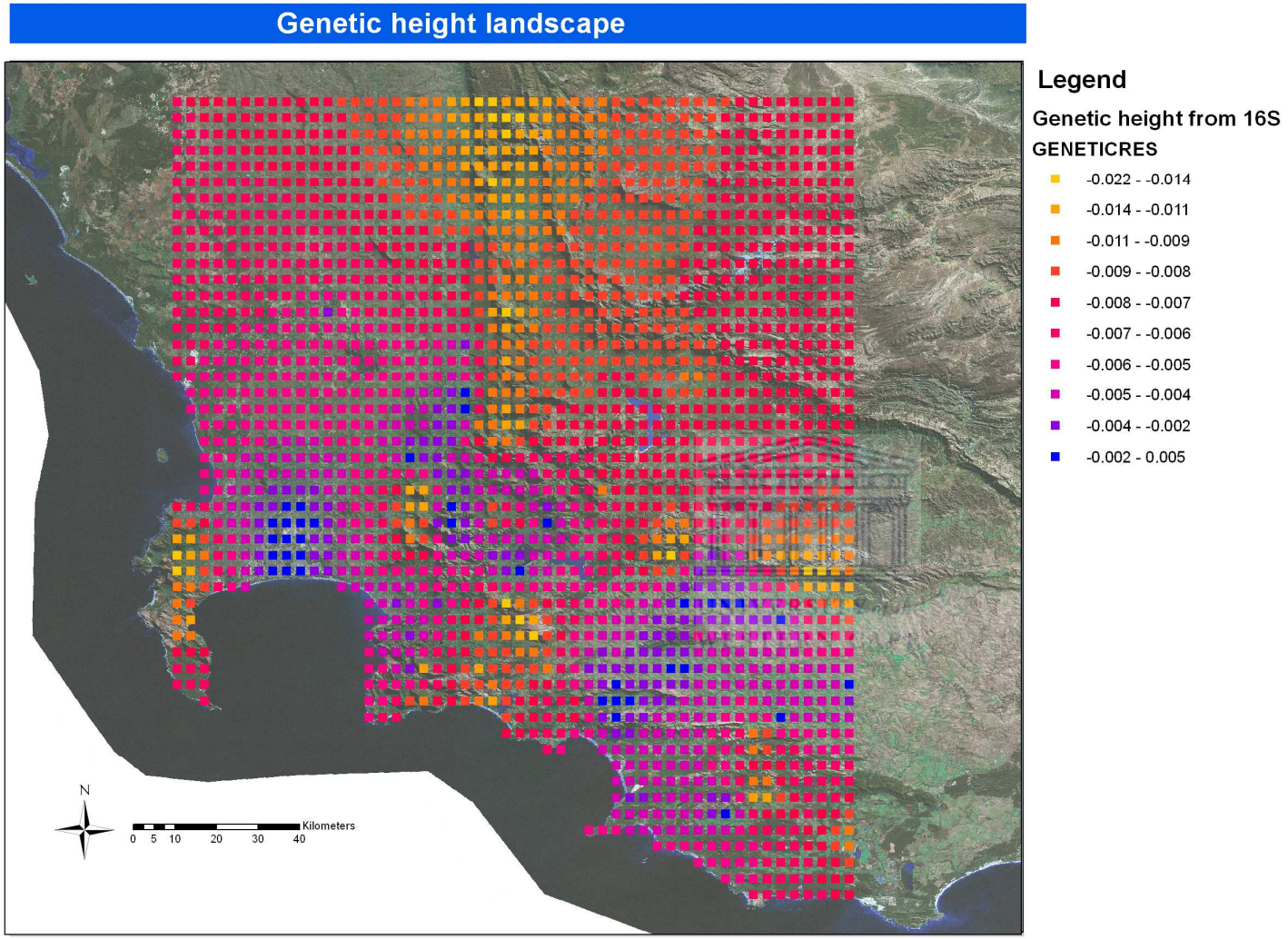

Figure 69. Location in geographical space of genetic diversity of all *Arthroleptella* species as measured by residual genetic distances plotted as genetic 'heights'.

The distribution of genetic diversity across the spatial landscape is displayed in Figure 69. The most obvious pattern is that genetic diversity coincided with mountainous regions. This is to be expected given the association of *Arthroleptella* with mountainous regions. However, within the mountainous regions the patterns are not uniform with patches of higher diversity found on certain mountains e.g. Cape Peninsula, Jonkershoek, Limietberg and Riviersonderend.

AMOVA

The groups used in the AMOVA analyses did not share any haplotypes. Haplotype frequencies for each group are given in Table 54. When all populations were included and grouped into Chirping and Clicking clades, the AMOVA framework returned significant and high values of all fixation indices ($F_{SC,}$ $F_{ST\ and}$ $F_{CT)}$ for all sources of variance (among groups, among populations within groups, and within populations respectively, Table 54, Appendix 3). These results indicate lack of gene flow between the Chirping and Clicking clades and lack of gene flow between *A. sp.* A East, *A. bicolor, A. drewesii, A. sp.* B, *A. landdrosia, A. lightfooti, A. rugosa, A. villiersi* and *A. subvoce* as different populations. Variation between groups (Chirping vs. Clicking) was highest with less variation among populations and little variation within populations as defined by the species epithets above.

Table 54. Haplotype counts for each taxon group as used in AMOVA analyses.

Species	Haplotype count
A. landdrosia	17
A. drewesii	3
A. bicolor	5
A. subvoce	6
A. villiersi	16
A. lightfooti	6
A. rugosa	5
A. sp. A	13
A. sp. B	8
A. sp. C	7

Table 55. Summary of AMOVA results.

	Source of variation	Fixation Indices	D.F.	Sum of squares	Variance components	% variation	p
Click + Chirp	F_{SC}	0.75148	1	780.235	19.2772	72.11	0.01173 ± 0.00335
(All)	F_{ST}	0.93070	8	369.954	5.60192	20.96	<0.000001 ± <0.000002
	F_{CT}	0.72114	76	140.8	1.85263	6.93	<0.000001 ± <0.000003
	Total		85	1290.988	26.73174		
Chirp	F_{SC}	0.78275	1	73.598	6.45637	62.11	0.34115 ± 0.01680
(subset of All)	F_{ST}	0.91769	1	27.785	3.08252	29.66	<0.000001 ± <0.000002
	F_{CT}	0.62114	24	20.533	0.85556	8.23	<0.000001 ± <0.000003
	Total		26	121.889	10.39444		
Click	F_{SC}	0.65878	1	72.298	2.22256	24.69	0.01173 ± 0.00335
(subset of All)	F_{ST}	0.74304	5	196.3	4.46533	49.61	<0.000001 ± <0.000002
	F_{CT}	0.24693	52	120.266	2.31281	25.7	<0.000001 ± <0.000003
	Total		58	388.864	9.0007		
A. sp. A split E and W	F_{SC}	0.73544	1	72.298	2.1497	24.11	0.03324 ± 0.00514
(subset of Click)	F_{ST}	0.79923	6	225.285	4.97543	55.81	<0.000001 ± <0.000002
	F_{CT}	0.24113	51	91.281	1.78982	20.08	<0.000001 ± <0.000003
	Total		58	388.864	8.91495		
A. sp. A split E and W &	F_{SC}	0.74163	1	72.298	2.35117	26.58	0.02444 ± 0.00495
Houwhoek split from *A.*	F_{ST}	0.81031	7	232.673	4.81627	54.45	<0.000001 ± <0.000002
landdrosia (subset of	F_{CT}	0.26581	50	83.893	1.67786	18.97	<0.000001 ± <0.000003
Click)	Total		58	388.864	8.8453		

When the analysis was run for the Clicking and Chirping clades separately the patterns of variation were different. In an analysis of the Chirping clade with *A. lightfooti* and *A. villiersi* (as close sister species) in one group and *A. rugosa* in another group, the pattern of variation resembled that for the entire genus (most between groups, less between populations and little within populations) and was significant for variation between populations and within populations but not between groups. In contrast, an analysis of the Clicking clade showed most variation between populations, less variation between groups and even slightly less variation within populations (see Table 55).

The F_{ST} values for this comparison were high and significant (see Table 56, Appendix 3). This pattern was maintained when *A. landdrosia* was split to represent the Houwhoek as a separate population and also when *A. sp.* A was split to represent the western and eastern populations separately. In all three Clicking clade AMOVA analyses, all sources of variation were significant (see Table 56).

Table 56. Summary of number of significant pairwise F_{ST} comparisons and exact tests.

Clicking + Chirping (All)										
	A. sp. C	*A. landdrosia*	*A. drewesii*	*A. sp.* A	*A. sp.* B	*A. bicolor*	*A. subvoce*	*A. villiersi*	*A. lightfooti*	*A. rugosa*
Sample size	7	17	3	13	8	5	6	16	6	5
F_{ST} sig. comparisons	7	7	7	7	7	7	7	7	7	7
Sig. exact tests	9	7	3	7	8	2	7	9	7	7
Proportion sig. exact tests	1	0.78	0.33	0.78	0.89	0.22	0.78	1.00	0.78	0.78
Chirping (subset of All)										
	A. villiersi	*A. lightfooti*	*A. rugosa*							
Sample size	16	6	5							
F_{ST} sig. comparisons	2	2	2							
Sig. exact tests	2	2	2							
Proportion sig. exact tests	1	1	1							
Clicking (subset of All)										
	A. sp. C	*A. landdrosia*	*A. drewesii*	*A. sp.* A	*A. sp.* B	*A. bicolor*	*A. subvoce*			
Sample size	7	17	3	13	8	5	6			
F_{ST} sig. comparisons	6	6	6	6	6	6	6			
Sig. exact tests	6	4	2	4	6	2	4			
Proportion sig. exact tests	1	0.67	0.33	0.67	1.00	0.33	0.67			
A. sp. A split E and W										
	A. sp. C	*A. landdrosia*	*A. drewesii*	*A. sp.* A W	*A. sp.* A E	*A. sp.* B	*A. bicolor*	*A. subvoce*		
Sample size	7	17	3	6	7	8	5	6		
F_{ST} sig. comparisons	7	7	6	7	7	7	6	7		
Sig. exact tests	7	5	2	5	4	7	2	4		
Proportion sig. exact tests	1	0.71	0.29	0.71	0.57	1.00	0.29	0.57		
A. sp. A East & West & *A. landdrosia* "Houwhoek"										
	A. sp. C	*A. landdrosia*	Houwhoek	*A. drewesii*	*A. sp.* A W	A. sp. A E	*A. sp.* B	*A. bicolor*	*A. subvoce*	
Sample size	7	11	6	3	6	7	8	5	6	
F_{ST} sig. comparisons	8	8	8	8	8	8	8	8	8	
Sig. exact tests	8	6	4	3	7	4	7	1	4	
Proportion sig. exact tests	1	0.75	0.50	0.38	0.88	0.50	0.88	0.13	0.50	

Pairwise comparisons of FST from all populations and exact differentiation tests are shown in Table 56. When all populations were included and when Chirping and Clicking clades were analysed separately, the pattern of significant difference varied slightly depending on the constitution of the Landdrosia clade. When the *A. sp*. A populations were split into west and east groups and when *A. landdrosia* "Houwhoek" was treated separately the among population variation increased and within population variation decreased.

Most population comparisons, no matter the grouping scheme, were significant (see summary results in Table 55). Complete results of all F_{ST} values, AMOVA and exact tests are presented in Appendix 3. In the exact tests of the Clicking clade *A. bicolor* was not significantly differentiated from *A. landdrosia, A. drewesii*, the A. sp. A or *A. subvoce*; *A. drewesii* was not differentiated from *A. landdrosia*, the *A. sp*. A or *A. subvoce;* and *A. landdrosia* was not differentiated from *A. bicolor*.

A similar pattern was obtained when the *A. sp*. A and Landdrosia clades were considered separately with the following additional differences:

- *bicolor* did not have a significantly different F_{ST} to *A. drewesii*;
- In the pairwise exact tests *A. drewesii* was not different from either the *A. sp*. A West or *A. sp*. A East;
- *A. bicolor* was not different from either the *A. sp*. A West or *A. sp*. A East;
- *A. subvoce* was not different from the *A. sp*. A East;
- the Houwhoek population was not different from *A. drewesii, A. sp*. A East, *A. bicolor* or *A. subvoce*.

The west and east *A. sp*. A populations were differentiated from each other, as was the Houwhoek population from *A. landdrosia*.

SAMOVA

When 16S samples were analysed using SAMOVA the results were significant for all tests as shown in Table 57. The following values are presented: proportionate within population genetic variation (F_{SC}), proportionate genetic variation due to population structure (F_{ST}), and proportionate genetic variation due to groups of populations (F_{CT}). Unless indicated otherwise, F_{SC}, F_{ST}, and F_{CT} are significant (at alpha = 0.0001).

Table 57. Results of SAMOVA tests.

Data set	Number of groups	F_{SC}	F_{ST}	F_{CT}	p
All	2	0.84404	0.91837	0.47662	
	3	0.83266	0.91877	0.51461	
	4	0.92735	0.96349	0.49741	
	5	0.79389	0.90118	0.52053	
	6	0.80643	0.90434	0.50582	
	7	0.74761	0.89660	0.59032	
	8	0.79940	0.90146	0.50877	
	9	0.78554	0.89888	0.52850	
	10	0.73396	0.89146	0.59202	
	11	0.75810	0.89264	0.55617	
	12	0.75810	0.89264	0.55617	
	13	0.64880	0.88682	0.67772	
	14	0.66634	0.88928	0.66816	
	15	0.61739	0.88531	0.70025	
	16	0.62102	0.88953	0.70852	
	17	0.61624	0.88916	0.71117	
	18	0.67787	0.88951	0.65701	
	19	0.54832	0.88525	0.74596	
	20	0.50865	0.88441	0.76474	
	21	0.61353	0.88403	0.69992	
	22	0.49658	0.88442	0.77041	
	30	0.30164	0.88140	0.83018	
Chirping	2	0.94927	0.98838	0.77093	F_{CT} 0.01075±0.00265
	3	0.95053	0.98553	0.70748	F_{CT} 0.00196 ± 0.00136
	4	0.86667	0.97818	0.83633	<0.00001
	5	0.82571	0.97757	0.87131	F_{SC} 0.00293 ± 0.00000
	6	0.83428	0.97582	0.85407	F_{SC} 0.00391 ± 0.00185
	7	0.77034	0.97564	0.89395	F_{SC} 0.07918 ± 0.00000
	8	0.74900	0.97534	0.90177	F_{SC} 0.01564 ± 0.00442
	9	0.68156	0.97507	0.92172	F_{SC} 0.16813 ± 0.01123
	10	0.48485	0.97423	0.94998	F_{SC} 0.34115 ± 0.01070
	11	0.54545	0.97399	0.94278	F_{SC} 0.49951 ± 0.00276
	12	0.48000	0.97377	0.94956	F_{SC} 1.00000 ± 0.00000
	13	0.05882	0.97356	0.97191	F_{SC} 0.09091 ± 0.00000
	14	0.64000	0.97340	0.92612	F_{SC} 0.33236 ± 0.01329
	15	0.00000	0.97287	1.00000	F_{SC} 1.00000 ± 0.00000
	16	0.00000	0.97275	1.00000	F_{SC} 1.00000 ± 0.00000 F_{CT} 0.00196 ± 0.00136
Clicking	2	0.76414	0.84148	0.32790	
	3	0.73378	0.84490	0.41739	
	4	0.66936	0.84089	0.51879	
	5	0.72807	0.84604	0.43381	
	6	0.57790	0.83845	0.61727	
	7	0.54714	0.83720	0.64051	
	8	0.55458	0.83646	0.63283	
	9	0.53568	0.83676	0.64842	
	10	0.53811	0.83602	0.64499	
	11	0.46354	0.82563	0.67497	
	12	0.36170	0.82592	0.72728	
	13	0.45726	0.93686	0.88367	

Data set	Number of groups	F_{SC}	F_{ST}	F_{CT}	p
	14	0.62802	0.93687	0.83029	
	15	0.22261	0.93583	0.91745	
	16	0.52850	0.93641	0.86512	
	17	0.33888	0.93551	0.90245	
	18	0.25962	0.93534	0.91267	
	19	0.52465	0.93592	0.86519	
	20	-0.04346	0.81575	0.82342	
	21	0.32014	0.93395	0.90285	
	22	0.42103	0.93473	0.88727	
	23	0.11552	0.93478	0.92626	
	24	-0.65475	0.93373	0.95995	
	25	0.07037	0.93366	0.92864	
	30	0.11419	0.93298	0.92434	F_{SC} 0.10753 ± 0.00780 F_{CT} 0.00293 ± 0.00164

From the maximum values of F_{CT} in Table 57 it can be seen that the number of groups represented by the data is at least 30 for the entire genus, at least 15 for the Chirping group and 24 for the Clicking clade. Note however that version 1.0 of SAMOVA does not seem designed to typically allow more than 20 groups to be tested and regularly crashed when attempting more than 30 groups. Examination of F_{SC} in the Chirping clade indicates that intra-population variation tends to non-significance at more than eight groups. This may indicate that no more than eight genetically homogenous populations were sampled even though splitting the Chirping clade into more than eight groups increased between group variance. The groups retrieved by SAMOVA for the Chirping and Clicking clades are listed in Table 58 and Table 59 respectively.

Table 58. Localities represented by number of groups (15) maximising F_{CT} in the Chirping clade.

Group	Constituent localities	Mountain range	Species represented
1	Back Table	Table Mountain	*A. lightfooti*
2	Aasfontein	Soetanysberg	*A. villiersi*
2	Steenboksberg	Steenboksberg	*A. villiersi*
3	Jonkershoek	Jonkershoek	*A. villiersi*
4	Greyton	Riviersonderend Mountain	*A. villiersi*
5	Caledon_1	Caledon Swartberg	*A. rugosa*
6	Caledon_2	Caledon Swartberg	*A. rugosa*
7	Paarl_Rock	Paarl Mountain	*A. villiersi*
8	Franschhoek Pass	Franschhoek	*A. villiersi*
9	Steenbras	Koëlberg	*A. villiersi*
9	Betty's Bay	Kogelberg	*A. villiersi*
10	Silvermine	Silvermine	*A. lightfooti*
11	Babilonstoring	Babilonstoring	*A. villiersi*
12	Cape Point	Cape Point	*A. lightfooti*
13	Cecilia	Table Mountain	*A. lightfooti*
14	Landdroskop	Landdroskop	*A. villiersi*
15	Bredasdorpberg	Bredasdorpberg	*A. villiersi*
15	Agulhas	Soetanysberg	*A. villiersi*

Table 59. Localities represented by number of groups (24) maximising F_{CT} in the Clicking clade.

Group	Constituent localities		Species represented
1	Jonkershoek	Jonkershoek	*A. landdrosia*
1	SE Simonsberg_1	Simonsberg	*A. landdrosia*
2	Houwhoek	Houwhoek	*A. landdrosia*
3	Jonaskop	Riviersonderend Mountain	*A. sp.* A West
4	Amanzi	Riviersonderend Mountain	*A. sp.* A West
5	Oudebosch	Kogelberg	*A. sp.* C
5	Steenbras 1	Koëlberg	*A. sp.* C
6	Die Galg_1	Riviersonderend Mountain	*A. sp.* A East
6	Die Galg_2	Riviersonderend Mountain	*A. sp.* A East
7	Kanonberg	Riviersonderend Mountain	*A. sp.* A East
8	Du Toitskloof	Du Toitskloof	*A. sp.* B
8	Fizantakraal	Du Toitskloof	*A. sp.* B
8	Zachariashoek	Klein Drakenstein	*A. sp.* B
9	Aasvogelberg	Aasvogelberg	*A. sp.* B
10	SE Simonsberg_2	Riviersonderend Mountain	*A. landdrosia*
11	Landdroskop	Riviersonderend Mountain	*A. landdrosia*
12	Amanzi_1	Riviersonderend Mountain	*A. sp.* A West
12	Jonaskop	Riviersonderend Mountain	*A. sp.* A West
13	Sneeugat	Sneeugat	*A. subvoce*
14	Delheim	Simonsberg	*A. landdrosia*
15	Babilonstoring	Babilonstoring	*A. drewesii*
15	Fernkloof	Kleinriviersberg	*A. drewesii*
16	Observation Peak	Limietberg	*A. bicolor*
17	Grootwinterhoek W.A.	Grootwinterhoek	*A. subvoce*
18	Helderberg	Helderberg	*A. landdrosia*
18	Swartboskloof	Swartboskloof	*A. landdrosia*
19	Twistwyk_1	Riviersonderend Mountain	*A. sp.* A East
20	Twistwyk_2	Riviersonderend Mountain	*A. sp.* A East
21	Waterval NR	Waterval Mountain	*A. bicolor*
22	Groenlandberg	Groenlandberg	*A. landdrosia*
23	Bettys Bay_1	Kogelberg	*Kogelberg*
24	Bainskloof	Bainskloof	*A. bicolor*

DIVA results

The DIVA analysis ran to completion without any limitations. Ronquist's (1997) and Kodandaramaiah's (2010) recommendation to include sister groups in DIVA analyses would not inform the current analysis as the sister genus *Natalobatrachus* occurs more than 500 km away from any extant *Arthroleptella* in a very different habitat. The DIVA analysis provided an optimal dispersal vicariance solution that indicates that most populations arose by vicariant events (terminal nodes 1-9 and 11 in Figure 70). Five dispersal events were invoked for *A. villiersi* which is very widespread compared to all other *Arthroleptella* species. See terminal node 10 in Figure 70. The nodes in Figure 70 have been colour coded to show adjacent geographical areas as follows: green: Hottentots-Holland mountain complex, Helderberg and Groenlandberg and Houwhoek mountains; orange: Kogelberg mountains; blue: Kleinriviersberg. The dispersal of *A. villiersi* is invoked as the

most parsimonious solution as the alternative requires the dispersal of three different species into the areas occupied by *A. villiersi* coupled with the subsequent extinction of these three species in their previously occupied areas.

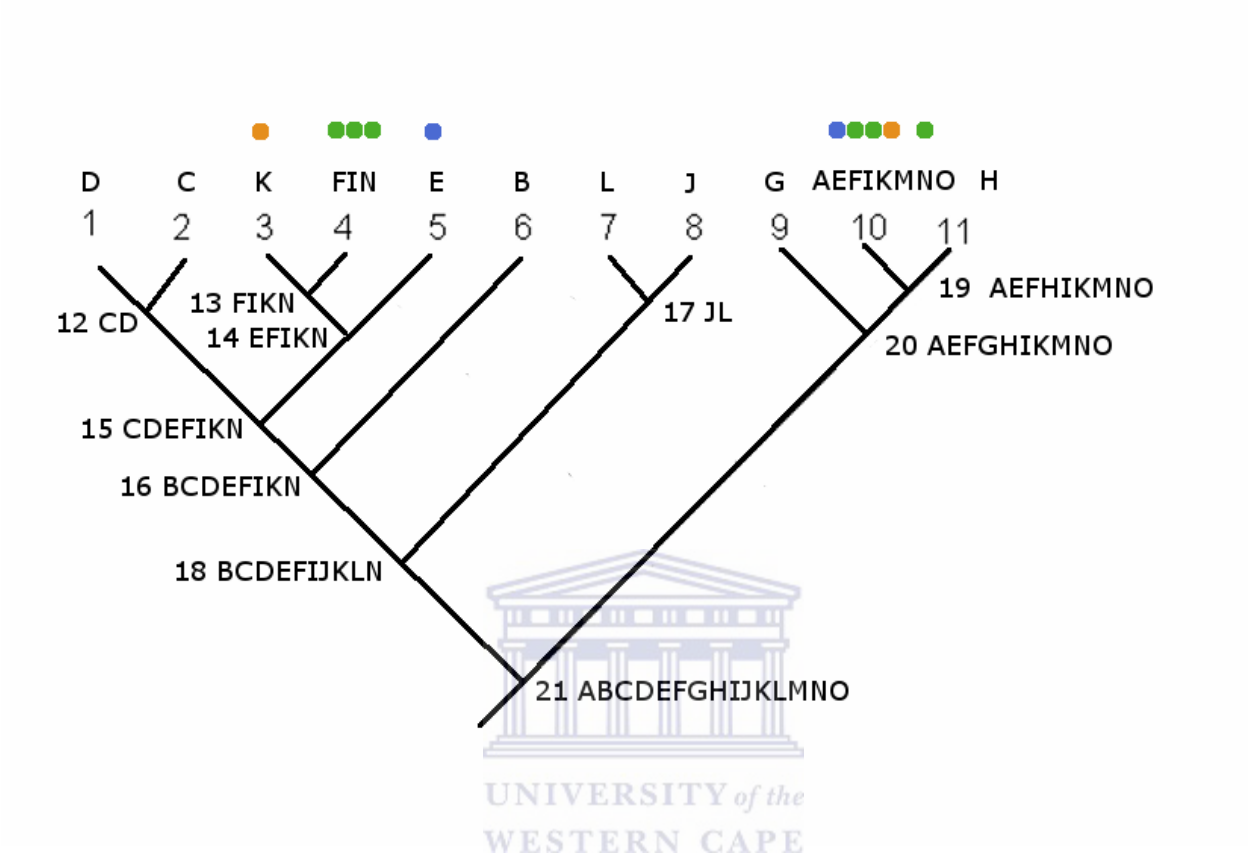

Figure 70. Area cladogram derived from DIVA analysis. Coloured dots represent areas occupied by *A. villiersi* (represented by node 10). Areas are labeled as in Table 60.

Table 60. Areas used in dispersal-vicariance analysis. See Figure 2 for locations of mountain ranges.

Area label	Area
A	Breadasberg & Soetanysberg mountains
B	Du Toitskloof mountains
C	E. Riviersonderend Mountains
D	W. Riviersonderend Mountains
E	Kleinriviersberg
F	Hottentots-Holland and adjacent mountains
G	Caledon Klein Swartberg
H	Peninsula (Table Mountain chain)
I	Helderberg
J	Grootwinterhoek Wilderness Area
K	Kogelberg
L	Limietberg
M	Bredasdorpberg
N	Groenlandberg & Houwhoek mountains
O	Groot Drakenstein & Franschhoek mountains

3.4.6 Species inferences

Species delimitations

Objective methods to identify population boundaries based on the source of genetic variance, gene flow and spatial location such as AMOVA and SAMOVA are useful in indicating species boundaries. These methods, which use measures such as F_{ST}, provide insights not readily discernable from genetic divergence *per se* (Ferguson 2002). Several of the clades identified by these procedures above were interpreted to represent species. Firstly I will deal with the clades that clearly represent species and then the clades with more complex patterns.

1. *A. rugosa*. This taxon is geographically isolated from all others and is strongly genetically divergent, morphologically distinct and has very distinct calls.

2. *A. sp.* B. This taxon is genetically the sister taxon to the Landdrosia clade + *A. sp.* A clade and it is consistently retrieved as a separate monophyletic clade. Its calls distinguish it from *A. bicolor* and the Landdrosia + *A. sp.* A clade but it is morphologically difficult to distinguish from members of the Landdrosia + *A. sp.* A clade. The single individual of *A. sp*. B sequenced from Aasvogelberg (labelled AVK) is intriguing as although it always clusters with the Du Toitskloof Mountain populations it is quite different from them genetically (16S: 2.0 %, 12S: 2.6 %). This is of further interest as the Aasvogelberg population is located in a very isolated seep surrounded by large tracts of steep, rocky mountains. Further samples from this area are required to properly place this population in a systematic context.

3. In the Lightfooti-villiersi clade there are two described species *A. lightfooti* and *A. villiersi*. There are several features of these frogs, their distribution and evolutionary history that argue for the maintenance of these two taxa as two separate species. They are separated by a large geographical gap of unsuitable habitat viz. the Cape Flats which are unconsolidated quaternary marine sands and the clay-rich lowland to the north of the Cape Flats. The time since the point of coalescence between *A. lightfooti* and *A. villiersi* is approximately 13 ± 1.1 Ma which represents a significant period of separate evolution.

   There are three ways interpret the species status *A. lightfooti* and *A. villiersi.*
   One is that they represent separate species in spite of the nested placement of *A. lightfooti* within the *A. villiersi* clade. Although reciprocal monophyly provides a clear indication of separate species distinctions recently evolved species such as those arising via divergent

selection would not be recognized under methods that rely on the reciprocal monophyly (Knowles & Carstens 2007). I hypothesise that the reason for this arrangement is that *A. villiersi* is the extant ancestor of *A. lightfooti* where *A. lightfooti* represents a peripatric bud off the widely distributed *A. villiersi*. This pattern of paraphyly of the ancestral species is likely to occur when peripheral populations speciate from a widespread ancestor (Talbot & Shields 1996; Hedin 1997; Funk & Omland 2003; Sites & Marshall 2004). It is hypothesised that *A. villiersi* dispersed to the peninsula about 13 Ma (there was a major sea level regression 11.2 Ma, see Figure 3) and that these two lineages diverged during the subsequent marine transgressions.

Two, is to recognise *A. villiersi* as polyphyletic with respect to *A. lightfooti* given the paraphyly in some of the phylograms (Figures 34, 35, 36 and 38) and that *A. villiersi* comprises more than one species. However, there is no clear evidence based on call characters or geographical distribution to delineate the exact nature or spatial boundaries separating *A. villiersi* (excluding *A. lightfooti*) into more than one species. Increased sample sizes and a more comprehensive spatial sampling may shed light on this possibility.

Three is that *A. villiersi* be considered a junior synonym of *A. lightfooti* as *A. lightfooti* is nested within *A. villiersi* but *A. lightfooti* Hewitt 1926 has nomenclatural priority over *A. villiersi* (Hewitt 1935). It is possible that populations of *A. lightfooti* and *A. villiersi* may have coalesced and separated repeatedly over the several more recent marine regressions and transgressions (Figure 3). This is not indicated by the 13 Ma mean estimated coalescence time but the error about this estimate (lower 95% highest posterior density bounds from 0.8 Ma) makes this a possibility. This process is compatible with the incomplete lineage separation as a cause of the paraphyletic arrangement shown by the 16S mitochondrial sequences. If it can be shown that *A. lightfooti* have been in recent genetic contact and are still genetically compatible then this viewpoint will be corroborated. I suggest that more data be obtained to test this hypothesis before carrying out this systematic action.

The Landdrosia clade was the most complex clade examined in this study and was consistently polyphyletic. Within the clade there are two described species *A. landdrosia* and *A. drewesii*. *Arthroleptella drewesii* is a reasonably clear and well-defined species based on its allopatric distribution, distinct calls and neatly monophyletic tree position in the mitochondrial and combined nuclear and mitochondrial trees (Channing *et al.* 1994, Turner

et al. 2004, Turner & Channing 2008) and this study. However, there is a clade sister to *A. landdrosia*, which is recognised here as *A. sp.* C. Although the genetic distance between *A. drewesii, A. sp.* C and *A. landdrosia* was not great there were no shared mitochondrial or nuclear RAG-1 haplotypes. In addition, there is a narrow but distinct geographic break in the distribution and genetic markers that corresponds with a band of shale that separates *A. sp.* C from *A. landdrosia*. The Hottentots-Holland mountains mark a second divide amongst lineages in at least five independent studies, reviewed in (Linder *et al.* 2010). These factors support the recognition of *A. sp*. C as a species.

4. The remaining constituents of the Landdrosia clade were subsumed in *A. landdrosia*. However, there was clear phylogeographic structure within this taxon with a separate clade consistently retrieved on the northern Jonkershoek and Simonsberg mountains. The populations from the Groenlandberg Mountain clustered separately with strong support as did the population from the Houwhoek Mountain. There is a substantial geographic barrier (an arid shale band) between the topotypical population of *A. landdrosia* (Landdroskop) and the Groenlandberg Mountain. However, the Groenlandberg and Houwhoek Mountains are very nearly continuous and are only separated by an approximately 2 km wide section of lower lying land that does not contain suitable *Arthroleptella* habitat. That there is any genetic divergence between the Groenlandberg and Houwhoek Mountain *Arthroleptella* populations is surprising but yet more surprising is that the Groenlandberg population has somewhat different calls and the Houwhoek population has markedly different calls to topotypical *A. landdrosia*. This seems to be a clear case of increasing isolation by distance with vicariant splitting due to lack of suitable habitat as it has shrunk over time as the CFK has dried. This pattern is supported by the results of the Mantel tests and autocorrelation tests performed on the Landdrosia clade. This pattern is interpreted as one of incipient speciation and both these populations may warrant recognition as separate species. Before this can justified it would be useful to increase very fine scale spatial (<= 1km) sampling between Landdroskop, Groenlandberg and Houwhoek Mountains.

5. The Bicolor clade contains only two described species *A. bicolor* and *A. subvoce*. Some phylogenies (16S Figure 35, Mt and Nu Figure 38) show a reciprocally monophyletic relationship between these two species. However, the presence of a very small, isolated population on the southern slopes of the Groot Winterhoek Mountains (Sneeugat north of the town of Tulbagh) renders *A. subvoce* paraphyletic with respect to *A. bicolor*. The tendency for the Sneeugat population to cause paraphyly in the *A. bicolor* and *A. subvoce*

clades is not surprising as the Sneeugat population is geographically intermediate between topotypical *A. subvoce* and *A. bicolor*. The likely reason for the paraphyly is incomplete lineage sorting in the mitochondrial line. Hybridisation as an explanation for the pattern, e.g. (Austin *et al.* 2003) is discounted as the Sneeugat population is not in current contact with other members of the Bicolor clade. The Sneeugat population should be regarded an evolutionary lineage in its own right as it is very isolated and shows differences in advertisement calls. Also there may be subtle morphological differences although current sample sizes preclude robust conclusions. The same three possible interpretations applied to the *A. lightfooti – A. villiersi* clade apply: recognition of *A. bicolor* and *A. subvoce* as separate species despite paraphyly; recognition of *A. subvoce* as polyphyletic; and considering *A. subvoce* a junior synonym of *A. bicolor* as *A. bicolor* (Hewitt 1926) has priority over *A. subvoce* Turner *et al.* 2004). *Arthroleptella subvoce* is clearly diagnosable as a separate taxonomic entity from *A. bicolor* based on call and morphological differences. The differences in advertisement calls between toptypical *A. bicolor* and *A. subvoce* would be remarkable if *A. subvoce* was to be considered a junior synonym of *A. bicolor* as *A. bicolor*. The geographic distribution of the populations in the *A. bicolor – A. subvoce* clade indicates that isolation by distance is responsible for the divergence. Further sampling of the Sneeugat population may provide a resolution to the current unsatisfactory systematic arrangement.

3.5 The influence of underlying geology and soils

There is a very tight coupling of *Arthroleptella* distribution with the Table Mountain sandstones (see Figure 9). As can be seen in Figure 9, almost all recorded *Arthroleptella* populations were on arenites which are primarily derived from TMS. In the few instances where moss frogs were not found on TMS, they were on granite-derived substrates. They are entirely absent from shale and shale-derived soils.

3.6 Morphology

3.6.1 External morphology

A total of 90 specimens were measured. Most specimens (80 individuals) were males as they are more easily located by their vocalisations. Females could only be collected opportunistically. The measurements of one female specimen were discarded as the individual was immature.

The results of the measurements of both males and females are presented in Table 61. Proportionate morphological characters are represented by the morphometric ratios in Table 62.

Table 61. Average linear morphological measurements (mm). Eyelid not measured in the *A. rugosa* male specimens.

Taxon	Sex	Head body	Head width	Snout	Eye	Eyelid	Inter-orbit	Inter-nares	Femur	Tibia	Foot	Toe	Leg	N
A. sp. A West	Male	13.0	5.1	1.3	1.5	2.0	2.1	1.4	4.8	5.1	3.3	5.7	18.9	5
A. sp. A East	Female	12.9	4.8	1.4	1.6	2.0	2.0	1.4	5.8	6.0	3.6	6.3	21.6	1
A. sp. A East	Male	13.4	5.1	1.2	1.4	2.1	2.2	1.3	5.1	5.4	3.5	6.2	20.3	6
A. bicolor	Female	13.3	4.8	1.1	1.6	1.8	2.4	1.2	4.8	4.7	2.6	5.3	17.4	1
A. bicolor	Male	12.2	4.7	1.1	1.3	1.9	2.0	1.2	4.6	4.4	3.1	5.5	17.5	3
A. drewesii	Female	17.3	6.4	1.3	1.8	2.1	2.4	1.5	6.6	7.1	4.3	8.0	25.9	1
A. drewesii	Male	13.2	5.3	1.2	1.3	2.2	2.5	1.3	5.1	5.4	3.5	5.9	19.9	2
A. sp. B	Female	14.5	5.0	1.3	1.3	1.8	1.9	1.1	4.6	5.0	2.6	5.3	17.5	1
A. sp. B	Male	12.9	4.9	1.2	1.5	2.0	2.1	1.3	4.7	4.8	3.1	5.4	18	4
A. sp. C	Male	14.3	5.4	1.3	1.5	2.1	2.1	1.3	5.1	5.5	3.4	6.1	20.2	7
A. landdrosia	Male	13.1	5.2	1.2	1.3	2.0	2.3	1.3	4.8	5.1	3.2	5.7	18.7	9
A. landdrosia "Houwhoek"	Female	12.2	4.3	1.1	1.4	1.8	1.8	1.1	4.5	4.4	3.1	5.3	17.4	2
A. landdrosia "Houwhoek"	Male	12.6	4.6	1.1	1.4	1.9	1.9	1.2	4.5	4.7	3.2	5.4	17.8	9
A. lightfooti	Male	13.4	5.1	1.2	1.4	2.0	2.0	1.3	4.9	5.2	3.2	6.0	19.4	9
A. rugosa	Female	15.5	5.3	0.9	1.1	1.9	2.1	1.3	5.3	5.9	3.7	6.9	21.8	1
A. rugosa	Male	13.2	5.1	1.1	1.4	-	2.9	1.3	5.7	5.1	3.1	5.3	19.2	5
A. subvoce	Female	13.9	5.1	1.3	1.5	1.7	2.2	1.1	4.4	5.4	3.5	4.7	17.9	2
A. subvoce	Male	12.2	4.8	1.1	1.4	1.7	2.1	1.0	4.5	4.8	2.9	4.9	17.2	7
A. villiersi	Male	13.3	5.1	1.2	1.4	2.0	2.0	1.2	5.1	5.4	3.7	6.1	20.3	14

Table 62. Average morphological ratios. Key: HW = head width, HB = Head Body, FM =Femur, IO = Inter orbit, IN = Inter nares, SN = Snout, E = Eye, EL = Eye lid, LE = Leg.

Species	Sex	HW: HB	FM: HB	FM: Toe	HB: FM	IO: HB	IO: FM	IO: EL	IN: HB	IN: FM	SN: HB	SN: HW	SN: E	E: HW	HB: LE	HW: LE	N
A. sp. A West	Male	0.39	0.95	0.84	2.72	0.16	0.44	1.05	0.10	0.44	0.10	0.26	0.85	0.30	0.69	0.27	5
A. sp. A East	Female	0.37	0.97	0.93	2.23	0.15	0.34	1.01	0.11	0.34	0.10	0.28	0.82	0.34	0.60	0.22	1
A. sp. A East	Male	0.38	0.94	0.82	2.64	0.16	0.43	1.06	0.10	0.43	0.09	0.24	0.88	0.28	0.66	0.25	6
A. bicolor	Female	0.36	1.01	0.91	2.78	0.18	0.49	1.30	0.09	0.49	0.09	0.24	0.70	0.34	0.77	0.28	1
A. bicolor	Male	0.39	1.03	0.84	2.68	0.16	0.43	1.03	0.10	0.43	0.09	0.24	0.90	0.27	0.70	0.27	3
A. drewesii	Female	0.37	0.93	0.83	2.62	0.14	0.37	1.15	0.09	0.37	0.07	0.20	0.71	0.28	0.67	0.25	1
A. drewesii	Male	0.40	0.94	0.87	2.58	0.19	0.48	1.14	0.10	0.48	0.09	0.23	0.95	0.24	0.66	0.27	2
A. sp. B	Female	0.35	0.91	0.87	3.16	0.13	0.41	1.03	0.08	0.41	0.09	0.25	1.00	0.25	0.83	0.29	1
A. sp. B	Male	0.38	0.98	0.88	2.74	0.16	0.45	1.07	0.10	0.45	0.09	0.24	0.80	0.30	0.72	0.27	4
A. sp. C	Male	0.38	0.92	0.84	2.80	0.14	0.41	0.98	0.09	0.41	0.09	0.23	0.86	0.27	0.71	0.27	7
A. landdrosia	Male	0.40	0.95	0.84	2.73	0.18	0.49	1.20	0.10	0.49	0.09	0.23	0.93	0.25	0.70	0.28	9
A. landdrosia "Houwhoek"	Female	0.36	1.03	0.86	2.69	0.15	0.40	1.02	0.09	0.40	0.09	0.25	0.79	0.31	0.70	0.25	2
A. landdrosia "Houwhoek"	Male	0.37	0.97	0.84	2.77	0.15	0.42	1.00	0.10	0.42	0.09	0.25	0.85	0.29	0.71	0.26	6
A. lightfooti	Male	0.38	0.95	0.82	2.72	0.15	0.41	1.00	0.10	0.41	0.09	0.24	0.86	0.28	0.69	0.27	9
A. rugosa	Female	0.34	0.90	0.77	2.92	0.13	0.38	1.07	0.09	0.38	0.06	0.17	0.87	0.20	0.71	0.24	1
A. rugosa	Male	0.39	1.12	1.06	2.33	0.22	0.51		0.10	0.51	0.09	0.22	0.81	0.27	0.68	0.27	5
A. subvoce	Female	0.37	0.82	0.96	3.18	0.16	0.50	1.30	0.08	0.50	0.09	0.26	0.88	0.29	0.78	0.29	2
A. subvoce	Male	0.39	0.95	0.93	2.70	0.17	0.46	1.22	0.08	0.46	0.09	0.23	0.77	0.30	0.71	0.28	7
A. villiersi	Male	0.38	0.94	0.86	2.63	0.15	0.39	0.99	0.09	0.39	0.09	0.24	0.87	0.28	0.66	0.25	14

Statistical comparison of morphology

Statistical comparisons were made between the two main call clades *viz*. the Clicking and Chirping clades. Sample sizes for the Chirping and Clicking clade were 28 and 52 respectively, allowing robust conclusions at this phylogenetic resolution. These results are presented in Table 63. Comparisons were made between males of different species (see pairwise comparisons Table 64). The sample sizes for females (9 females across all species) were too small to allow meaningful statistical comparisons. Sample sizes for comparison of males from different species was also small which diminishes the robustness of any conclusions that may be drawn from these results. For this reason, a finer spatial categorisation of samples by mountain ranges was not attempted.

Table 63. Morphological differences (linear morphometric measures and morphometric ratios) between the Clicking and Chirping clades from Wilcoxon rank sum test comparisons. All other measurements and ratios were not statistically significant between Clicking and Chirping clades.

Character	P-value
Femur	0.0007
Tibia	0.023
Toe	0.017
Leg	0.0014
HB:Femur	0.012
Inter orbit:Femur	0.044
Inter nares:Femur	0.044
Head body:Leg	0.01
Head width:Leg	0.0023
Inter orbit:Eyelid	0.035

Table 64. Morphological differences (linear morphometric measures and morphometric ratios) between species from pairwise Wilcoxon rank sum test comparisons. All other pairwise species comparisons for all other measurements and ratios examined were not statistically significant.

Character	Pairwise comparison	P-value
Femur	*A. rugosa : A. landdrosia*	0.032
Femur	*A. rugosa : A. lightfooti*	0.043
Tibia	*A. sp.* A East *: A. landdrosia*	0.04
Tibia	*A. sp.* C *: A. landdrosia*	0.022
Foot	*A. sp.* A East *: A. landdrosia*	0.02
Toe	*A. subvoce : A. lightfooti*	0.042
Toe	*A. sp.* A East *: A. lightfooti*	0.038
Leg	*A. sp.* A East *: A. landdrosia*	0.0432
Leg	*A. villiersi : A. landdrosia*	0.0086
Leg	*A. villiersi : A. subvoce*	0.0403
HeadWidth:Leg	*A. villiersi : A. sp.* B	0.0195
HeadWidth:Leg	*A. villiersi : A. subvoce*	0.0009

Notable among the results in Table 62 is that *A. villiersi* had the longest legs relative to head body length (although *Arthroleptella sp.* A East and *Arthroleptella drewesii* were only marginally shorter). Frogs in the Chirping clade as a whole had significantly longer femur and overall leg length than those in the Clicking clade (Table 63). It is also noteworthy that all of the significant comparisons, except the inter orbit:eyelid ratio comparison between calling clades, involved an aspect of leg length.

Hewitt (1935) stated that interorbital distance could be used to distinguish *A. villiersi* from *A. lightfooti* with the distance in *A. lightfooti* being "sub-equal to or not much exceeding" the breadth of the upper eyelid whereas this ratio in *A. villiersi* is 1 ½ to two. The morphological ratios in Table 62 do not support this difference *viz.* 1 in *A. lightfooti* and 0.99 in *A. villiersi*. Comparisons of interorbital to eyelid ratios did not result in significant differences between any species pair although this ratio is significantly larger in the Clicking clade than in the Chirping clade (Table 63).

The data presented here do not support Hewitt's 1935 contention that eye size is relatively larger in *A. lightfooti* than *A. villiersi*. In general, eye and eyelid size were not found to be useful diagnostic characters.

Qualitative morphological characters

Several qualitative morphological characters have been used for diagnosing species in *Arthroleptella*. The following is a brief treatment of the state of these characters in the current study.

The state of the toe tips has been described as slightly swollen in *A. bicolor* (Hewitt 1926), simple in *A. villiersi* (Hewitt 1935), slightly enlarged tips in *A. bicolor* (Hewitt 1935), slightly swollen in *A. landdrosia* (Dawood & Channing 2000), and slightly expanded in *A. drewesii* which is also stated to have slightly expanded finger tips (Channing *et al.* 1994). In this study, the degree of expansion of the finger and toe tips was found to be variable and difficult to quantify. The only discernable pattern was that the Clicking clade tended to have more expanded digit tips (although these are less well developed in *A. bicolor* and yet less in *A. subvoce*) than members of the Chirping clade, although *A. rugosa* has swollen digit tips.

Dorsal skin texture (mostly influenced by the presence of raised structures that may contain glands) has been mentioned as a character by Hewitt (1926; 1935), Channing *et al.* (1994), Dawood & Channing (2000), Turner *et al.* (2004) and Turner & Channing (2008). In this study, in which an

expanded sample of live specimens was examined, this was found to be a very variable character that only reliably separates *A. rugosa* from all other species. It seems that there are differences in this character between sexes: females, with the exception of *A. rugosa*, have smoother dorsal skin. There may be seasonal variation in this character too as the degree of skin rugosity changed in captive specimens. Further work is required to assess the significance of this variation.

Another glandular character is the presence of a broken glandular ridge extending from the upper lip at the angle of the jaw to arm insertion as described for *A. drewesii* (Channing *et al.* 1994). This character may have some utility as a distinguishing character: certain members of the Landdrosia clade have well developed upper jaw ridges that may form a continuous line (*A. drewesii*, *A. sp.* C) whereas they are less well developed in topotypical *A. landdrosia*, and *A. bicolor*; broken up into separate lumps in *A. villiersi* and *A. lightfooti;* moderately developed to forming a continuous line in *A. rugosa* and poorly developed in *A. subvoce.* Again there is considerable intra-specific variation in this character which requires further data and analysis.

Hewitt (1926) noted that *A. bicolor* has the "postero-ventral portion of the thigh with conspicuous scale-like corrugation of the skin extending rather more than half the length of the thigh". Blister-like bumps (see Figure 71) in the pelvic and thigh areas were noted on many males of all species and on a female specimen of *A. sp*. B. However, this feature was not always discernable and may also be subject to temporal variation. The function of these bumps, presumably glandular tissue, deserves further investigation.

Figure 71. Ventral view of male of A. sp. C showing pelvic and thigh bumps.

The degree of development of the vocal sac is useful in distinguishing certain species: the vocal sac is well-developed with obvious lateral folds in *A. drewesii*, *A. landdrosia*, *A. sp*. A (East and West), *A. lightfooti* and *A. villiersi*. It is less well-developed in *A. subvoce*, *A. bicolor*, *A. sp*. B and *A. sp.* C although this character is rather variable in the last-mentioned.

Hewitt (1926) suggested that there are differences in stoutness (unmeasured) between *A. lightfooti* and *A. villiersi*. This is a difficult character to measure but there are differences in head-width relative to snout-urostyle length (Table 62). The differences are more useful in separating clades than individual species. There were no significant differences in relative head width between *A. lightfooti* and *A. villiersi*. In general, members of the Clicking clade have relatively broader heads and this may create the impression of greater ‘stoutness’.

Snout shape was found to be useful for several distinctions: it is rounded in *A. subvoce, A. rugosa, A. drewesii, A. landdrosia;* and pointed in *A. villiersi* and *A. lightfooti*. The snout profile in *A. lightfooti* appears to be blunter than *A. villiersi* in accordance with Hewitt’s 1935 description.

Arthroleptella sp. B appears to have a more depressed head but appropriate measurements to assess this subtle character such as the ratio of skull height to width have not yet been examined.

The inner and outer metatarsal tubercles are best developed in *A. rugosa.* The inner and outer metatarsal tubercles are well-developed in *A. lightfooti* and *A. villiersi* although the outer metatarsal tubercles are less well developed in *A. lightfooti*. In the Clicking clade, inner and outer metatarsal tubercles and subarticular tubercles are less prominent than in the Chirping clade. Differences in these characters between species within the Clicking clade are not clearly diagnostic.

Arthroleptella lightfooti and *A. villiersi* differ from all other species by the combination of their relatively long limbs, more pointed snouts (more pointed in *A. villiersi* than *A. lightfooti)* and relatively unexpanded digital terminals. *Arthroleptella rugosa* differs from all other species by its very warty appearance and the dark undersides of females. *Arthroleptella subvoce* is of very small size and has relatively large eyes. Members of the Landdrosia clade are difficult to separate morphologically.

3.6.2 Skeletal morphology

X-rays of 47 *Arthroleptella* specimens spanning the geographic range of the genus were examined for qualitative skeletal characters. Skeletal characters such as the shape of omosternum (Hewitt 1926; Poynton 1964) and various other characters of the shoulder girdle were not included in this study as they were difficult to observe in the still articulated specimens. Few differences were found apart from proportional leg differences and the more pointed snout of *A. villiersi*. Both of these differences were also detected by the external morphological measurements. This line of investigation was hampered by insufficient fine-scale resolution of the x-rays which were not clear enough to pick up small or subtle shape differences. Several x-rays suffered from differences in perspective which made reliable measurements impossible. Despite these caveats, there were indications that there are skeletal differences between certain *Arthroleptella* species. As already mentioned, there are differences in proportional leg bone lengths and overall skull shape and there may be differences in the shape of the ilea and other aspects of pelvis morphology such as a proportionately shorter pelvis in *A. rugosa* (compare Figure 72 and Figure 73 below).

Figure 72. Dorsal x-ray of *A. rugosa*.

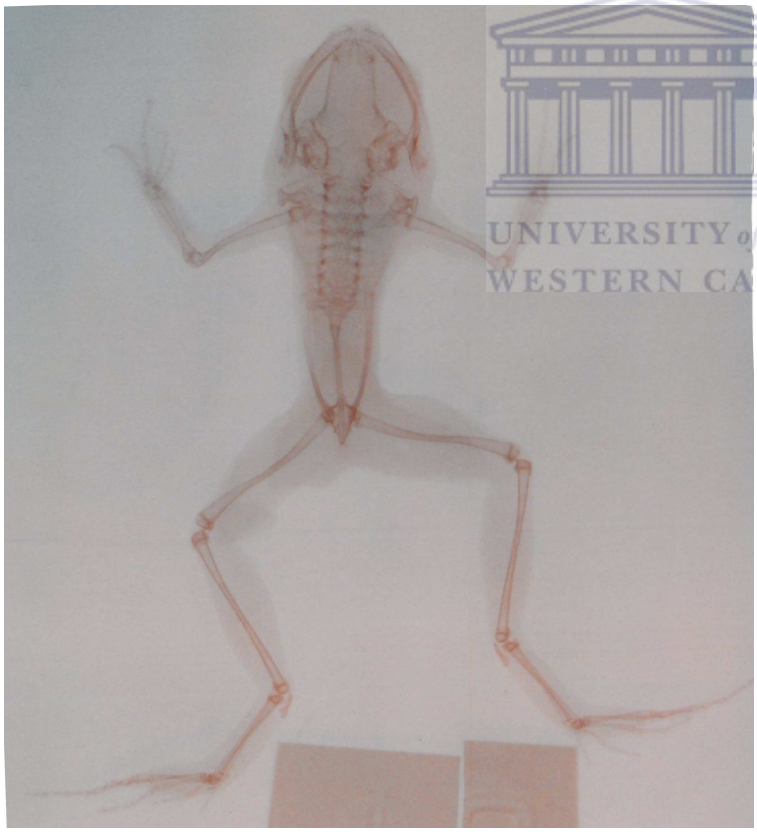

Figure 73. Dorsal x-ray of *A. villiersi*.

3.7 Habitat and conservation status

3.7.1 Habitat preferences

In all cases moss frogs were found concealed in vegetation or very occasionally under rocks in the case of *A. landdrosia* and *A. sp.* A East. The presence of some form of dense cover is required by all species although *A. villiersi* may be found in sparser vegetation they are still completely concealed within such vegetation. All individuals were always found on damp or wet substrates with some individuals of *A. villiersi* occasionally found on only slightly damp substrate although always close to damp substrate. They were never found in flowing water and if they jumped into flowing water in attempt at escape, they immediately swam to firm substrate.

The majority of moss frogs were recorded from fynbos vegetation and in particular southwest mountain fynbos (Figure 10). In the few cases where moss frogs were found in forest, these were surrounded by fynbos and the frogs occurred in the surrounding fynbos too. The species most regularly encountered in forest is *A. landdrosia* but within these forests is associated with rocky seeps. This species is more often found on steeper slopes, including cliff-faces than other species and is thus often associated with wet rocky environments rather than forest. Similarly, the presence of moss frogs in fynbos is postulated to be a result of the choice of perennially moist microhabitats which are currently mostly found in fynbos overlying TMS.

The patchy distribution of suitable habitat is the fundamental cause of the patchy distribution of these frogs. The patches of suitable habitat are not only widely dispersed in the landscape but are often very small and can only support small moss frog populations. The implications of the distribution and size of suitable habitat for moss frog populations and hence conservation status is discussed in the next section.

3.7.2 Conservation assessment

EOO & AOO

The extent of occurrence estimated by creating alpha hulls and minimum convex hulls resulted in polygons of identical topology. The shape and extents of the minimum convex hull polygons are shown in Figure 74.

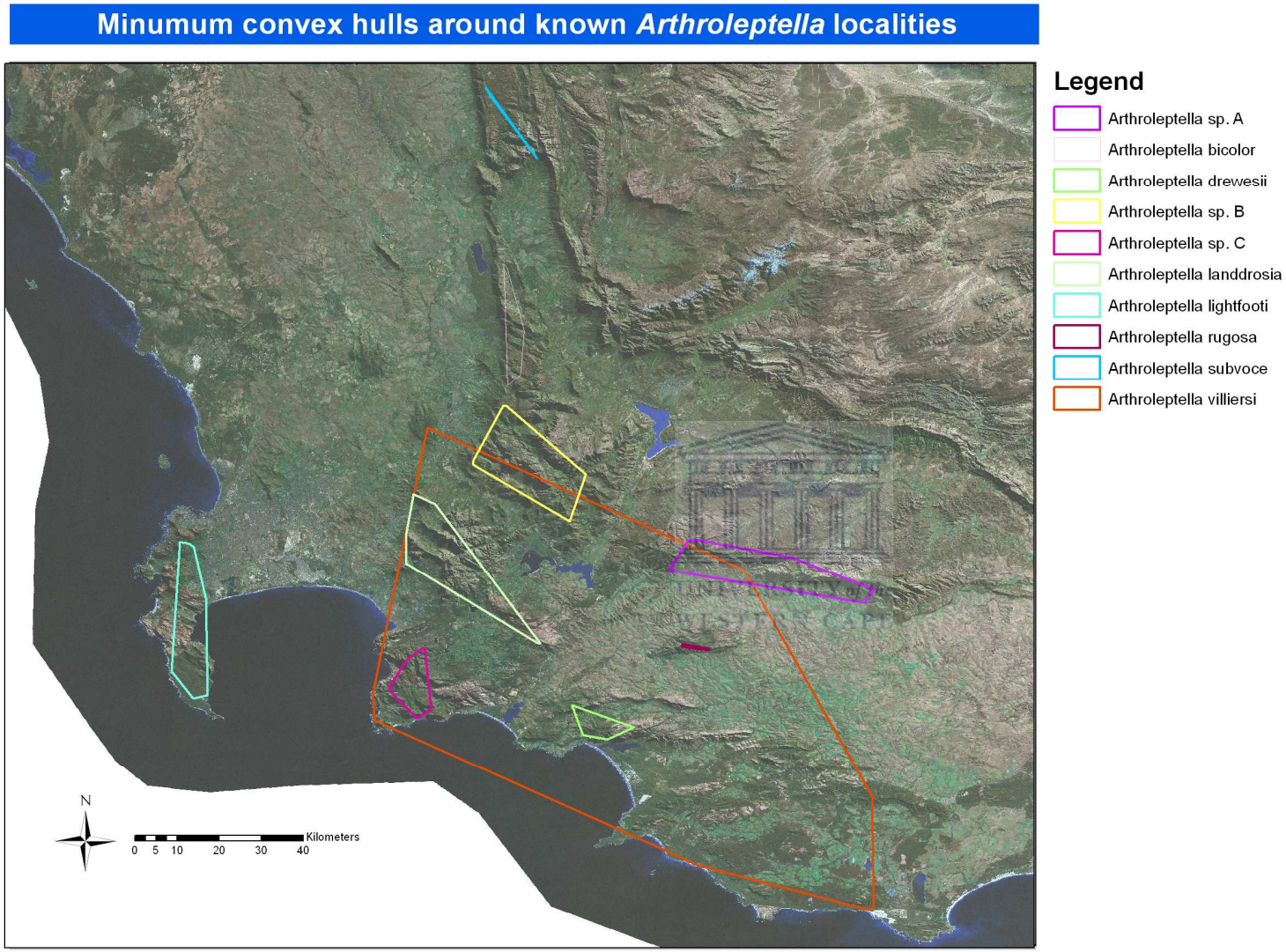

Figure 74. Minimum convex hulls based on known occurrences of *Arthroleptella* species.

The area of the minimum convex hull (Table 65) was the primary input for assessing the B criterion. As can be seen in the table there is a wide range in the EOO for different species ranging from over 6 000 km^2 to only 2.4 km^2. The AOO varies in exactly the same way as it was calculated as a fixed percentage (10 %) of EOO.

Table 65. Extent of occurrence (EOO) and area of occupancy (AOO) in km^2. AOO estimated as 10 % of EOO.

Species	EOO	AOO
A. sp. A East & West	272.7	27.3
A. bicolor	67.3	6.7
A. drewesii	32.4	3.2
A. sp. B	299.0	29.9
A. sp. C	82.1	8.2
A. landdrosia	287.3	28.7
A. lightfooti	199.4	19.9
A. rugosa	2.4	0.2
A. subvoce	3.3	0.3
A. villiersi	6382.7	638.3

Population size

Estimations of populations size were made only for *A. rugosa* as it was the only species where this was practical. The size of the known populations of *A. rugosa* is estimated at 400 adult individuals and the population over all suitable habitat is estimated to be around 1 000 adults. Dramatic population size changes occur as a result of fire, including local extinctions (see below) and in species such as *A. subvoce* and *A. rugosa* with a small number of populations this may cause the entire species population numbers to vary dramatically.

Time to maturity is not known for this genus. This is an important gap in our knowledge as rapid maturation may counter the effects of population reductions from the effects of fire. Given the genus exposure to fire over at least the last 3 million years it is predicted that frogs in this genus will mature rapidly.

Clutch size

Clutch size in *Arthroleptella* is the smallest of all South African frogs (M.J. Cunningham & C.L. Henderson, unpublished data) with recorded clutch sizes varying between 5 and 12 (Hewitt 1926;

Morgan *et al.* 1989; Dawood & Stam 2006; Turner & Channing 2008). Small clutch size is known to be a factor associated with amphibian population declines (Hero *et al.* 2005; Bielby *et al.* 2008) and hence is likely to increase extinction risk in *Arthroleptella*. However, Murray & Hose (2005) found that range size was more important than clutch size in population declines of Australian frogs. These two factors have however found to be related: Cooper *et al.* (2008) show that tropical frogs with small clutches have the smallest ranges and these two factors are significantly correlated in Hero *et al.*'s 2005 study. Small clutch size is expected to apply to all species of *Arthroleptella* and several species have very small ranges. The extinction risk posed by fire is likely to be exacerbated by small clutch size in the genus *Arthroleptella* as it will limit the ability of populations to recover. Taken in conjunction, these factors are expected to increase the overall risk of extinction to *Arthroleptella* species.

Disease

Several diseases have been implicated in amphibian declines across the world (e.g. (Berger *et al.* 1998; Kiesecker *et al.* 2001; Daszak *et al.* 2003). Prevalence of the widely implicated chytrid fungus (*Batrachochytrium dendrobatidis*) has not yet been determined for any *Arthroleptella* species. Terrestrial breeding mode is negatively associated with rapid or enigmatic amphibian declines (e.g. Hero *et al*. 2005). If this holds for *Arthroleptella* then exposure risk to this disease may be low. As the role of *B. dendrobatidis* and other diseases in this genus is unknown, disease was not used in the threat assessments.

3.7.3 Threats

The main threats to *Arthroleptella* are damage to habitat which provides permanently available surface moisture. This habitat is primarily threatened by short fire-return intervals (fires returning before populations have reached a sustainable size) and invasion by alien plants. The invasion of alien invasive woody vegetation increases the impact of fires which may led to more dramatic population fluctuations. The few populations that occur in low-lying coastal areas are threatened by housing development.

Fire

Although it was not the aim of the current study to quantity the effects of fire as threat to *Arthroleptella* populations some data were obtained that may shed some initial light on this factor. Six sites in Jonkershoek, supporting populations of moss frogs were monitored before and after a large fire in March 2009. Of these sites, two areas previously supporting *A. landdrosia* had no calling frogs in the breeding season after the burn. In the case of *A. villiersi*, the bigger seeps

supporting populations before burns continued to support only small numbers after the burn. No calling males were recorded at small seeps that had populations of *A. villiersi* prior to the fire. This indicates that *Arthroleptella* are directly affected by fire. Indirect effects of fire on habitat quality through desiccation and providing opportunities for alien plants to invade are also important.

Vulnerability of a habitat to fire is difficult to assess over short time scales but these limited observations in the field indicate that seeps in open exposed areas must be very wet throughout the year. This means they must remain very wet throughout the year or close to rocky outcrops which protect the seep from fire, and possibly to some degree from the wind, to support sustainable populations of moss frogs. Seeps that had burnt into the underlying organic matter (peat) were not found to be more affected than seeps that suffered more superficial burns.

Invasive alien plants

Invasive alien plants can be reasoned to threaten moss frogs by altering moisture regimes, increasing fuel loads for fires and altering plant species composition (upon which moss frogs may be dependent) these effects were not directly measured or tested in this study. Their effects will be explored in the Discussion.

Assignment to IUCN threat categories

Consideration of what is known about the distribution of *Arthroleptella*, their habitat requirements, clutch size and threats results in one species classified as Least Concern, seven species as Near Threatened, one species as Vulnerable and one species as Critically Endangered (Table 66). The species *A. villiersi* is categorised as Least Concern due to its wide range of occurrence, numerous populations and abundance of individuals in several populations (choruses exceeding 100 calling males have been recorded for *A. villiersi* and *A. lightfooti*). The seven Near Threatened species have small range sizes which put them at risk but these species have multiple populations which mitigates against local extinctions. In the case of *A. subvoce* and *A. rugosa* however the small number of populations and the small total population sizes mean that the effect of local population extinctions may have a dramatic effect on species persistence.

Table 66. IUCN threat classification of *Arthroleptella* species.

Species	Threat category	Justification
A. sp. A	NT	B1b(iii)+B2b(iii)
A. bicolor	NT	B1b(iii)+B2b(iii)
A. drewesii	NT	B1 b(iv ,v) c(iii,iv) + 2b(iv ,v) c(iii,iv)
A. sp B	NT	B1b(iii)+B2b(iii)
A. sp C	NT	B1b(iii)+B2b(iii)
A. landdrosia	NT	B1 ab(ii,iii) c(iv) + 2ab(ii,iii) c(iv)
A. lightfooti	NT	B1 ab(ii,iii,v) c(iv) + 2ab(ii,iii,v) c(iv)
A. rugosa	CR	B1ab(i,ii,iii,v) c(iv) + 2ab(i,ii,iii,v) c(iv)
A. subvoce	VU	B1ac(iv)+2ac(iv)
A. villiersi	LC	

Chapter 4. Discussion and conclusions

4.1 Calls

Considerable variation was detected in advertisement calls of *Arthroleptella*. The variation spanned individuals, populations and species groupings. A similar degree of variation in calls both within and among species of *Cophixalus* has been found over the mountainous areas of Australia (Hoskin 2004). The relationship between population distribution and the evolution of advertisement calls is discussed below.

To distinguish cause and effect in the process of speciation, the temporal sequence of call divergence relative to genetic divergence must be considered. Insights into this process are provided by comparison of sympatric and allopatric species pairs. Despite evidence that calls diverge to a greater degree in sympatry than in allopatry, e.g. (Littlejohn 1965; Littlejohn 1977), Vences & Wake (2007) found bioacoustic character displacement in sympatry to be rare. In the case of *Litoria ewingii* and *L. verreauxii* reported by Littlejohn (1965), a subsequent and more extensive survey, revealed that variation in allopatric populations equalled that in sympatric populations (Smith *et al.* 2003). Vences *et al.* (2002) and Vences & Wake (2007) suggest that bioacoustic differentiation in frogs is not the primary event leading to reproductive isolation of incipient species and rather invoke allopatric speciation. Character displacement as a mode of call divergence is not considered to be important in the evolution of *Arthroleptella* due to the largely allopatric arrangement of divergent populations. The finding by Funk *et al.* (2009) that male call divergence and associated preference by females in Amazonian *Physalaemus* explains genetic divergence better than geographical barriers is not found to be the case with *Arthroleptella* as call differences are highly linked to geographic location and thus the relative effects of geography and behaviour cannot be easily separated. It is thus argued that the variation among *Arthroleptella* populations is a result of their isolated and patchy spatial distribution rather than a cause of the genetic distinctions among populations.

4.1.1 Advertisement calls and speciation in *Arthroleptella*

Stabilising selection is predicted by the Recognition Concept but is not predicted by sexual selection theory and Polakow *et al.* (1995) suggest that stabilising selection be used as a null hypothesis for tests of sexual selection on mating signals. Information on the stability or variability of advertisement calls in their role as mating signals is required for understanding the evolution of advertisement calls and their relationships to species boundaries.

There is little evidence of stabilizing selection in *Arthroleptella* as there is much diversification of calls even over small geographical distances (see the significant differences between populations in (Tables 23 to 34). Most *Arthroleptella* populations occur allopatrically and there does not appear to have been any opportunity for reinforcement selection (*sensu* Mayr 1942) to have led to the diversification in advertisement calls. The only cases of sympatry always involve *A. villiersi* of the Chirping clade and single species of the Clicking clade (depending on geographical location, see Figure 7). The calls of *A. villiersi* do not appear to differ in the presence of a member of the Clicking clade from those of *A. villiersi* where it occurs in isolation although the known number of independent sympatric locations is too small to allow statistical analysis.

The pattern in *Arthroleptella* appears to be one in which populations have diverged geographically, primarily by vicariant events (evidence for this process is discussed below). Advertisement calls of these isolated populations have diverged in allopatry as evidenced by the numerous call and genetic differences between populations. This raises the question of what causes changes in a feature as critical to the maintenance of mate recognition as the advertisement call? The microhabitat of the various populations of *Arthroleptella* does not appear to differ in any obvious structural way that would strongly influence the transmission of the advertisement call. However, this anecdotal observation has not been tested by measuring sound transmission in the various microhabitats and may warrant an empirical investigation to see if this may be a causal factor.

Another possible cause that has been invoked for changes in mate recognition systems in allopatry is the effect of genetic drift (e.g. Funk *et al*. 2009). The underlying mechanism is that the male advertisement call changes due to drift in the genes controlling the form of the advertisement call. Furthermore in small populations there is not only a greater opportunity for stochastic events (e.g. genetic mutations) and small population sampling effects (including founder effects) for male calls to change but also greater freedom for these changes to be successful. This is because in the new small populations females will be faced with reduced choices if many or all the males in the new population have a divergent call. This is similar to the situation as proposed by Kaneshiro (1976) and known as the Kaneshiro model. In this model there may be directional effects on mate choice among females for ancestral versus derived male signals such that females in derived populations are less choosy than females in ancestral populations (Kaneshiro 1976). A distinction between Kaneshiro's model and the model I propose here is that, at least initially, females mate with divergently signalling males by lack of choice rather than by being less discriminating. Subsequent evolution of signals and preference for particular signals may evolve through both sexual and natural selection.

An interesting conclusion derived from a test of the Kaneshiro model is that females from two populations with divergently signalling males are both likely to prefer ancestral males but would not prefer the males from the other derived population and so will be isolated from each other if they come into sympatry, even though both derived populations may be capable of merging with the ancestral population (Ödeen & Florin 2002).

Booksmythe *et al.* (2008) have shown that mate choice is influenced by distance between the signaller and respondent as well as quality of the signal in fiddler crabs. This is likely to be the case in moss frogs too, where it is energetically expensive for females to make their way through the very dense vegetation to a distant male in preference to a nearby male. Arak (1988) and Bishop *et al.* (1995) have shown that female *Epidalea calamita* and *Hyperolius marmoratus* respectively, prefer louder calls irrespective of the sound pressure level at source so closer males will be preferred if the females perceive their call to be louder even if there are louder males further away. This increases the probability of a proximal male with a divergent call achieving a successful mating and thus perpetuating the divergent call if it is heritable.

It would be very informative to know what role female mate choice has in facilitating novel variations in advertisement calls in newly isolated populations. It would be interesting to compare female mate choice to progressively more geographically distant populations of the various populations of the Clicking clade. Unfortunately testing female mate choice in *Arthroleptella* will be very difficult as *Arthroleptella* remain concealed so I have relied on the degree of male call variation among populations to hypothesise that females have coevolved their signal reception along with the changes in male advertisement calls.

The complex structure of the calls of most *Arthroleptella* species, with the exception of *A. lightfooti* and *A. villiersi*, may also contribute to variability simply by providing more opportunity for variation. There may be social interactions mediating call variation (Given 2005; Wells & Schwartz 2007) even though aggressive calls were not analysed in this study and it is possible that parts of the advertisement call may have multiple functions (e.g. Backwell 1988) or that there may be various call types contained in the concept of advertisement call as employed in this study (e.g. Passmore 1977).

The variability in advertisement calls encountered in this study calls into question the applicability of the bioacoustic phylogeny method employed by Wollenberg *et al*. (2007) to this study. In their

study, call measures were coded as characters for use in generating a phylogenetic tree. However, there is a lack of clear, defensible and consistent criteria for coding call measures, including dealing with the variability in these measures. This precluded the generation of a bioacoustic phylogeny for *Arthroleptella*.

4.2 Molecular evidence

4.2.1 The origin of *Arthroleptella*

The finding that *Arthroleptella* is the sister genus to *Natalobatrachus* in this study is in agreement with the phylogeny presented by Frost *et al.* (2006) but does not agree with phylogenies presented by Scott (2005) and Dawood & Stam (2006). Both Scott (2005) and Dawood & Stam (2006) retrieve *Anhydrophryne* as the sister genus of *Arthroleptella*. However, Dawood & Stam (2006) did not include *Natalobatrachus* in their phylogeny and thus did not place this genus relative to *Arthroleptella*. Scott (2005) included *Natalobatrachus* but did not include any nuclear gene fragments in her analysis.

The position of *Natalobatrachus* as sister genus of *Arthroleptella* has several important implications for the evolution of *Arthroleptella*. Firstly, the date of the split between *Natalobatrachus* and *Arthroleptella* is suggested to be 33 Ma (Figure 36). This means that *Arthroleptella* had its origins in the late Oligocene, a period when conditions were generally much wetter and warmer than at present and tropical forest vegetation predominated most of the Southern Africa (Coetzee & Rogers 1982). This suggests that the common ancestor of *Arthroleptella* and *Natalobatrachus* was a forest dwelling frog as *Natalobatrachus* still is.

Secondly, there is a large straight-line geographical distance between extant populations of *Arthroleptella* and *Natalobatrachus* of approximately 1 000 km; see distribution maps in Minter *et al.* (2004). This gap is situated of over the southern Cape from the eastern end of the Riviersonderend Mountains to just east of the Bashee River in the Eastern Cape. Such a wide gap between two sister genera demands explanation.

Genetic diversity in *Arthroleptella* increases from east to west. This pattern is consistent with higher frog species diversity in the western CFR (and higher diversity of frogs endemic to the CFR) and provides an insight into the evolution of the genus as a whole. There are also strong differences in species richness from the western to the eastern CFR amongst plants (Cowling & Lombard 2002; Linder & Hardy 2004). Clades across several reptilian, mammalian and insect groups converge in the western CFR and in contrast the eastern CFR show a shallower phylogenetic structure (Tolley *et*

al. 2006; Swart *et al.* 2009; Tolley *et al.* 2009). This pattern has been ascribed to the reduction in rainfall over the eastern CFR versus a more stable western CFR where frontal rain was maintained during periods of cooling (Cowling *et al.* 2009). I suggest that the western CFR has functioned as a refugium for *Arthroleptella*. A similar role has been suggested for many of the fynbos plants which also show a pre-Miocene antiquity: Verboom *et al.* (2009) suggest that it is the moister, cooler, and less seasonal environments of the Cape Fold mountains that have acted as refugia for the long-term persistence of fynbos.

Tolley *et al.* (2009) show that gene flow within three lizard species is lowest in the west but is also low in the eastern CFR despite a shallow phylogenetic structure in the east. They suggest that this may be due to "very recent limitations on gene flow and possibly retention of ancestral polymorphisms, rather than homogenisation of populations through contemporary gene flow." A restriction on gene flow between the eastern and western CFR is postulated to have arisen within *Strongylopus grayii* (Pyxicephalidae) because it breeds at different times of the year, matching the different rainfall regimes of these two regions (Tolley *et al.* 2010a).

Extinction in the Gouritz River which flows through the gap in the eastern CFR has been invoked to explain the distribution of the fish *Pseudobarbus* (Swartz *et al.* 2009).

Low diversity and extinctions in the examples discussed above correspond spatially with the geographical gap in the distribution of *Arthroleptella* and its sister genus *Natalobatrachus*. This suggests that the same process may be responsible for this gap and I propose that the geographic gap between *Arthroleptella* and *Natalobatrachus* is due to a historical extinction in the *Arthroleptella* lineage over the eastern CFR. I propose that the substantial aridification of the eastern CFR drove this extinction. The aridification was a result of the cooling of the oceans starting 33 Ma and increasing from 10-5 Ma with concomitant drops in ocean level of up to 120 m (Siesser 1980; Hendey 1983a; Barker *et al*. 2007). The drop in sea level would have exacerbated the decrease in rainfall over the interior of the of the eastern CFR due to the massive southward expansion of the Agulhas bank (up to 200 km) as this bank will have decreased rainfall as rainfall decreases with distance from the coast as the coastal mountains act to create a rain shadow (annual rainfall at George, 8 km inland, is 726 mm (South African Weather Bureau) whereas Oudtshoorn basin, 52 km inland, receives between 150 mm and 250 mm (Thompson *et al*. 2009)). This effect would be particularly severe in a potential mountain refuge such as the Great Swartberg range which would have been up to 400 km inland at the glacial maxima. The Great Swartberg is unsuited

to either *Arthroleptella* or *Natalobatrachus* and neither genus is represented on this extensive mountain range.

Poynton (1964, p. 137) states that “It is possible that the "Cacosterninae" had a secondary radiation in southern Africa, if not an origin there, and probably at least *Arthroleptella* and *Anhydrophryne* are autochthonous.” Van der Meijden *et al.* (in press) show that the basal pyxicephalids are widespread lowland fauna from central Africa and infer that the diversity of species in southern Africa is likely to have been due to dispersal and subsequent specialisation and speciation, particularly in southern Africa.

4.2.2 Speciation and phylogeographic patterns of evolution in *Arthroleptella*

Gene trees and species trees

A number of studies have noted that gene trees do not necessarily represent species trees (Pollard *et al.* 2006; Edwards *et al.* 2007; Edwards 2009; Leaché *et al.* 2009) and that discord between different genes is widespread when examining entire genomes (Pollard *et al.* 2006). The aim of this study was to describe the species trees using the information embedded in the gene trees and other lines of evidence. As the gene trees recovered in this study are not topologically identical, an integrative process is needed to approximate the species tree. This is can be achieved in a number of ways. A very widespread approach is to concatenate gene sets e.g. (Gadagkar *et al.* 2005). This approach has been criticised on the grounds that it may lead to misleading species tree interpretations and over-confidence in the resulting trees (Edwards 2009). However, although Edwards (2009) lists a substantial array of techniques that may overcome the failings of the concatenation approach, each of these has pros (e.g. applicable to many loci) and cons (e.g. not generating species branch lengths). At the time of the writing of this study there was no consensus in this newly emerging set of methods.

In this study, interpretation of clades identified using the tree building approaches as species was augmented by examining gene flow through the use of F_{ST} and related statistics. The latter techniques provided valuable insight into the cases of paraphyly (between *A. bicolor* and *A. subvoce;* and *A. lightfooti* and *A. villiersi*) in the mitochondrial gene trees and widespread polyphyly in the nuclear gene trees. The gene flow criterion comes closer to directly addressing the species concept that I have used in this study than reciprocal monophyly. Edwards (2009) suggests that gene tree monophyly be abandoned as a criterion for delimiting species (especially with mitochondrial genes) due to its conflation of patterns and criteria for diagnosibility at the level of genes and species in addition to over or under-resolving clades as species.

The concatenated mitochondrial phylogenetic tree did not differ much from each of the individual mitochondrial marker trees. The concatenated nuclear and mitochondrial phylogeny yielded a very similar arrangement to the mitochondrial phylogeny. This was due to strength of the signal in the mitochondrial data rather than to congruence in phylogenetic topologies as the nuclear trees were very different from the mitochondrial trees.

Phylogeny and the number of species recognised

At the conclusion of this study the following species of *Arthroleptella* were recognised:

- *A. bicolor*
- *A. drewesii*
- *A. landdrosia*
- *A. lightfooti*
- *A. rugosa*
- *A. subvoce* (provisional, possibly a synonym of *A. bicolor*)
- *A. villiersi* (provisional, possibly a synonym of *A. lightfooti*)
- *A. sp.* A (includes both western and eastern populations)
- *A. sp*. B
- *A. sp*. C

Species accounts are provided in Appendix 1. Several of these species assignments contain significant population structuring and require further investigation as they may still obscure cryptic species. In particular, *Arthroleptella sp*. A is problematic as the eastern and western populations of this grouping do not always from monophyletic clades and are not always retrieved as each other's closest relatives. Examples of such paraphyly are displayed in Figure 35 and Figure 36. However, the geographical arrangement of *A. sp*. A East and *A. sp*. A West on the Riviersonderend mountains provided no clues as to the nature of any historical or current geographical barrier. This disjunction may be the result of small population size, genetic drift and/or low vagility (Irwin 2002). Although the resolution of the timing of the coalescences of the four sampled locations of these populations is still considered coarse it is interesting to note that the time to coalescence of the two eastern populations exceeds that of the two western populations. This is even though the former are geographically separated by 7.6 km and the latter by 17.6 km. These taxa certainly require further investigation.

The various populations currently assigned to *A. landdrosia* display considerable phylogenetic structuring and a very patchy distribution. It is possible that this taxon is polyphyletic. At present I do not have sufficient information to consider the divergent Simonsberg/Jonkershoek and Groenlandberg/Houwhoek populations (analysed as *A. landdrosia* "Houwhoek") as separate species although the call differences displayed by the Houwhoek population are intriguing. A finer scale investigation of the nature of these differences among geographically isolated populations, with an emphasis on finding geographically intermediate populations, may clarify these relationships and may shed light on the relationship of this species to *Arthroleptella sp.* C to which it is closely related.

The currently described and undescribed species of *Arthroleptella* referred to in this study are genetically different and are not in immediate geographic contact. Furthermore they are not likely to make contact again due to anthropogenically induced climate change and even more so from anthropogenic landscape change due to agriculture and urbanization with all its concomitant infrastructure. These latter effects had a profound impact on much more mobile anurans such as *Bufo bufo* (Ray *et al.* 2002) and *Rana temporaria* (Hitchings & Beebee 1997). Thus the *Arthroleptella* populations assessed as species in this study are expected to evolve separately at least for the foreseeable future. The likelihood that these populations may become extinct in the short to medium term is the subject of the next section and is dealt with in detail in the section on Conservation.

Poynton (1964) synonymised *A. bicolor* and *A. bicolor villiersi* with *A. lightfooti* based on his finding, using a large sample of specimens, that the variation in the skeletal characters used to diagnose *A. bicolor* and *A. villiersi* was too great to be of systematic use. Part of the reason for this erroneous conclusion was that the specimens of *A. lightfooti* that he examined may have included multiple species and this would tend to increase variability. The samples he used for comparison spanned the ranges of *A. lightfooti, A. bicolor, A. villiersi* and possibly also *A. sp.* C from Koeelbaai and *A. drewesii* from Hermanus.

An argument can be made for recognising fewer of these clades as species. This would result in the recognition of only *A. rugosa* and *A. lightfooti* (the name *A. lightfooti* has priority over *A. villiersi*) in the Chirping clade and only *A. bicolor, A. drewesii* (the name *A. drewesii* has priority over *A. landdrosia*), *A. sp.* A and *A. sp.* B in the Clicking clade. However, this arrangement would not be a satisfactory representation of the evolution of quite divergent and allopatric populations that have not been in contact for very lengthy periods of time. Even in the case of *A. lightfooti* and *A. villiersi*

which are genetically relatively close, there is evidence of morphological and behavioural differentiation. It would entirely obscure the distinctive phylogenetic position occupied by *A. sp.* B which is sister to the Landdrosia + A. *sp*. A clades. *Arthroleptella bicolor* is sister to the Landdrosia + *A. sp*. A clade. Assigning this taxon to either *A. bicolor* or *A. drewesii* would obscure the boundaries of those taxa and beg the question as to why *A. bicolor* or *A. drewesii* should be recognised and hence would revert to the situation where only a single species of *Arthroleptella* is recognised. This would be an obfuscatory conclusion given the evidence of presented here, and previously (Channing *et al.* 1994; Dawood & Channing 2000; Turner *et al.* 2004; Turner & Channing 2008) for the recognition of several species. Furthermore it would ignore the many differences among populations in advertisement calls, morphology and phylogenetic structuring. Such a conclusion would ignore regional biodiversity and its particular spatial arrangement and will not further the description of the South African amphibian biota or how it evolved. Another consequence of such an action is that it would provide no guidance for the conservation of this biodiversity.

On the other hand, extreme application of the Phylogenetic Species Concept could label almost every population of *Arthroleptella* (with the exclusion of several *A. villiersi* populations which share haplotypes or have very similar haplotypes) as separate species. If it could be shown that there is no gene flow between these populations and the probability of them sharing genes via dispersal is very low then such a conclusion may be merited. I do not think that there is sufficient data to support this approach yet.

4.2.3 AMOVA

The application of AMOVA showed very little evidence of gene flow between populations. The large amount of variation between populations in the Clicking clade when grouped into only a Landdrosia clade and a Bicolor clade indicates that the grouping levels (Landdrosia clade and Bicolor clade) do not adequately account for the variation among populations. This matches the pattern obtained in the phylogenetic trees in which genetic variation in the Clicking clade is high.

The pairwise exact tests produced a number of non-significant comparisons for two taxa in particular: *A. bicolor* and *A. drewesii*. The sample sizes for these two species are small (5 samples in 3 populations, and 3 samples in 1 population respectively). Fitzpatrick (2009) clearly demonstrated small numbers of groups in hierarchical permutation tests reduce power by prohibiting an alpha value of 0.05 or lower to be reached. Because population boundaries were unknown during the design of this study it was not possible to predict the numbers required for

adequately sampling populations. However, significant values of alpha were obtained for all other populations and are informative of hierarchical population structuring in *Arthroleptella*. In general, the AMOVA and SAMOVA analyses indicate that many sites represent distinct populations. This is in agreement with predictions based on the known biology and life history of this genus.

It is also worth bearing in mind that natural populations of the same species are unlikely to have the same value of F_{ST}, if only because they have different sizes (Weir & Hill 2002). Further evidence of the significance of population structuring comes from splitting groups based on geography, i.e. treating the eastern and western populations of *A. sp*. A and *A. landdrosia* "Houwhoek" population of the Clicking clade separately still yielded significant differentiation. This would be expected to reduce F_{CT} if it involved splitting individuals that actually came from the same population into different groups. The lack of significant differentiation of both *A. bicolor* and *A. drewesii* from the other populations is ascribed to a sampling artefact as it is in contradistinction to the evidence from the calls and other gene fragments which clearly indicate separation of these two species. Further sampling of these two species is required for population level analyses.

4.2.4 Barriers to population contact

The barriers that were derived from the application of the Monmonier maximum difference algorithm generally coincided with geographical features (see Table 53). However, there were a few instances where the barriers drawn by the Monmonier process needed further explanation. In the case of *A. rugosa*, the Monmonier algorithm shows a barrier through the distribution of *A. rugosa*. This is an artefact of the data used because there was no 16S sample of *A. rugosa* from the western region of the distribution so the Monmonier algorithm could not take the existence of that population into account.

Interestingly no barrier was identified between *A. subvoce* and *A. bicolor* after 11 steps (a number greater than the number of recognised species). This is surprising given the anomalous distribution of *A. subvoce*. It is the only species to occur north of the Berg River and is the only species with populations located in Northwest Fynbos. The small genetic divergence between *A. bicolor* and *A. subvoce* is the cause of the Monmonier Algorithm not identifying a gene-flow barrier even though there is a physical barrier (the Klein Berg River and a dry shale band) that separates *A. bicolor* and *A. subvoce.*

Similarly the Monmonier algorithm associates the populations of the eastern False Bay with *A. lightfooti* on the Cape Peninsula. *Arthroleptella villiersi* is isolated from its sister species *A.*

lightfooti by the Cape Flats and False Bay which separate the Peninsula outcrop of CFM from the Hottentots-Holland and Kogelberg ranges to the north-east. This area was repeatedly inundated by transgressional seas (forming an extension of False Bay) over the Cenozoic with transgressions of over 10 m occurring 20-12, 4-3 and 2.5-2 Ma (Siesser & Dingle 1981; Wigley & Compton 2006). Subsequent transgressions in the Quaternary have been of minor amplitude (< 10 m) (Hendey 1983a; Ramsay & Cooper 2002).

False Bay would have been exposed 35 Ma, 5 Ma and 18 Ka during major sea-level regressions (Dingle & Rogers 1972; Siesser & Dingle 1981; Hendey 1983a; Ramsay & Cooper 2002; Wigley & Compton 2006) providing a terrestrial connection between the Cape Peninsula and the inland CFM mountains and thus also between *A. lightfooti* and *A. villiersi* (Figure 75).

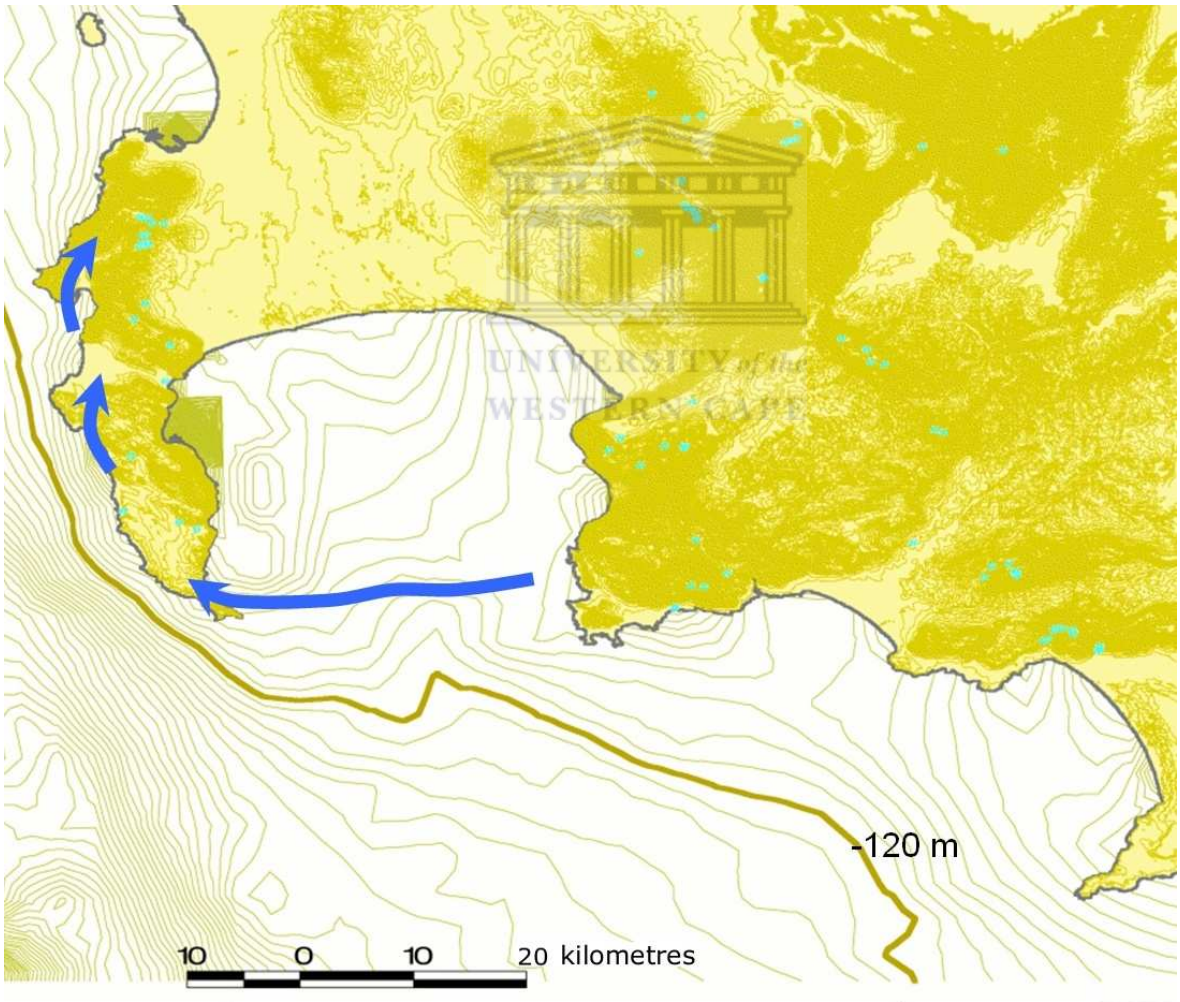

Figure 75. False Bay and Cape Flats. The -120 m isobath is shown to indicate the coastline at the lowest Cenozoic sea levels. Proposed historical corridor connecting Cape Peninsula *Arthroleptella* populations with those of the hinterland indicated by blue arrows.

The association of the *A. villiersi* population from the Kogelberg side of False Bay with *A. lightfooti* is ascribed to the historical spread of *A. villiersi* to the Cape Peninsula via the Kogelberg mountains,

through False Bay during one or more of the marine recessions. The last marine transgression, that persists to modern times, geographically isolated the population which gave rise to *A. lightfooti*.

4.2.5 Choice of method for phylogenetic reconstruction

There has been considerable debate in the literature on the best methods for phylogenetic reconstruction e.g. (Leaché & Reeder 2002; Holder & Lewis 2003; Kolaczkowski & Thornton 2004; Ronquist 2004). However, in practice the different methods of phylogeny construction very often produce the same topology (e.g. Makokha *et al.* 2007). This is to be expected in cases where the phylogenetic signal to noise ratio is high. Of the trees produced in this work the Bayesian method produced trees with the most resolution. This is an expected result as this method makes the most use of prior information. Like maximum likelihood methods it also makes use of models of nucleotide evolution which are not used in MP approaches. The MP trees obtained in this study, although often in topological agreement with the Bayesian trees, tended to be more poorly resolved. The phylogenetic trees obtained from direct optimisation using POY were not as instructive as the Bayesian trees but generally displayed better resolution and fewer polytomies than the MP approach. A practical limitation of POY is the inability to display branch lengths. This is unfortunate because branch lengths indicate the degree of genetic divergence and the POY trees were only evaluated on clade topology and the strength of the clades.

The combined mitochondrial and nuclear phylogeny is largely congruent with the mitochondrial phylogeny. The mitochondrial phylogenies provide greater resolution and indicate that the nuclear fragments used in this study do not contribute sufficiently to obtain a detailed phylogeographic representation of non-matrilineal inheritance. This is due to the tendency of the nuclear gene trees presented here to produce polytomies across populations. This indicates that these genes may not have diverged with speciation events due to genetic drift and raises the possibility that they are under stabilising selective pressure. The incorporation of non-coding nuclear sequences (such as beta-fibrinogen intron 7) in a phylogeny of this group may be instructive in testing this hypothesis. It is however noteworthy that Rag-1 yielded an *A. subvoce* clade and also an *A. lightfooti* clade, although in both these cases the sister taxa (*A. bicolor* and *A. villiersi* respectively) remained paraphyletic. A similar situation was found in members of the *Ambystoma tigrinum* complex (Weisrock *et al.* 2006).

Discord between mitochondrial and nuclear gene trees has been found in several studies (e.g. Moore 1995; Monsen & Blouin 2003). In recently evolved branches this discord is not surprising as lineage sorting may be incomplete and so species trees may not match gene trees e.g. (Tolley *et al.* 2006).

Moore (1995) argues that mitochondrial gene trees should track species trees better on short internodes. Monsen & Blouin (2003) also attribute such discordance to the demographic instability of frog populations and so such discord may be a common feature of frog genomes. The greater resolution afforded by the higher mutation rate in the mitochondrial sequences used in this study contributes to the identification of species trees and thus the phylogeny based on the combined mitochondrial sequences is taken to be a sufficient representation of the species relationships.

Many species-level phylogenies only sample a single individual per species (e.g. Smith *et al.* 2007). This work has shown that spatial sampling must be commensurate with the spatial scale at which the organism lives as previous studies did not capture the full array of species diversity within *Arthroleptella*. This is particularly the case with animals that are geographically restricted. The limited dispersal ability of these animals also influences the scale at which gene transfer occurs. This study has shown that there are restrictions and barriers to gene flow over very small geographic distances to the extent that individuals on the opposing sides of a valley through which a very small river runs may not be each other's closest relatives and valleys with strong flowing streams or rivers may represent significant barriers.

If I had not chosen to sample as many mountain ranges as I did, I would not have detected as much variation in genetic sequences and advertisement calls. Population-level analyses of gene flow at an even finer spatial scales in areas of dramatic population variation would lead to valuable insights into the mechanics of gene flow and speciation but fall beyond the scope of the current study. In a study of small plethodontid salamanders in Costa-Rica (Garcia-Paris *et al.* 2000) found high levels of genetic diversification over small distances. They ascribe this diversification to strong environmental heterogeneity which is influenced by the substantial elevational gradients in this area. The role of elevation has not been thoroughly investigated in *Arthroleptella* but appears to play a role in areas of sympatry between *A. villiersi* and members of the Clicking clade with *A. villiersi* tending to lower altitudes. Another factor invoked in the Garcia-Paris *et al*. (2000) study was small home ranges. This remains to be investigated in *Arthroleptella*.

4.2.6 Timing of cladogenic and speciation events

The evolution of the genus *Arthroleptella* has its origin in the late Eocene to early Oligocene (~33 Ma) with continuing evolution and speciation over the Miocene, Pliocene and Pleistocene. This range matches the period of radiation of the Cape flora (Linder 2005) and the radiation of the dwarf chameleon genus *Bradypodion* (Tolley *et al.* 2008) which have also undergone substantial speciation in the CFR. Diversification of the species complex clades in *Arthroleptella* occurred after

approximately 26, 22, 20, 18, 14, 13, 11 and 8 Ma (Figure 55). Within-species coalescence times are estimated to span from 9 to 0.1 Ma with 7 of these events occurring in the Pliocene (3.5-5) Ma (see Table 52). The majority of *Bradypodion* speciation events in the Western Cape also occurred over this period (Tolley *et al.* 2008). Notable environmental events over this time were major uplift at roughly 18 and 2.5 Ma (Partridge & Maud 1987) and a cooling and drying trend from 33 Ma (Barker *et al*. 2007). The cooling strengthened with the development of the Benguela current from 12 – 10 Ma (Siesser 1980) and intensified further over the past 3 Ma with increased cold water upwelling (Hendey 1983a). The pulses in climatic change, particularly the cooling and drying events appear to be roughly associated with speciation events but there is no way of rigorously testing this given the large margin of error around the coalescence time estimates. However, the modern distribution of *Arthroleptella* with very tight coupling to their patchy habitat indicate that past climate change would have a significant impact on the availability and suitability of habitat.

The current pattern of highly fragmented populations occupying a narrow range of habitats indicates that the current distribution is relictual and has formed by vicariant events. This constitutes a case of a genus having a relictual distribution within a vegetation type (fynbos), that it is itself relictual (Midgley *et al*. 2001). The climatic changes mentioned above would have directly affected moisture levels, vegetation structure, river patterns and flow and exposed novel substrates. Permanently high moisture levels are critical for the survival of moss frogs and the drying trend over the Western Cape would have drastically reduced suitable habitat. The drying of the Cape and the formation of the Mediterranean type climate resulted in a novel vegetation type evolving – fynbos. This would have placed further selection pressure on *Arthroleptella* as this vegetation type is very fire-prone and would act to reduce permanently moist areas that are required by *Arthroleptella*. The development of new rivers and increased flows with the two significant uplift events of the Cenozoic may also have created barriers to dispersal for *Arthroleptella* as would the exposure of soil types that do not have suitable moisture availability such as fine grained shales (Cowling *et al.* 2009). The role of tectonic uplift in conjunction with climatic fluctuations has been proposed to explain the phylogeographic patterns displayed by *Arenophryne rotunda* in the Shark Bay region of Australia (Edwards 2007).

4.3 Morphological evidence

Few significant differences in external morphological measurements were found between species. This was to be expected as the number of specimens collected (see Table 61) for morphological measurements was not sufficient to allow robust statistical comparisons amongst populations or species. This may be partly due to the large size of measurement error (between 0.1 and 0.5 mm)

relative to the expected size of the effect with an alpha of 0.05. The small size of all members of this genus limits the utility of many morphological measures and characters. This is due to the characters being physically hard to discern and measure even under magnification. There is also a tendency with miniaturisation for character simplification and loss (Hanken & Wake 1993; Yeh 2002; Agapow *et al.* 2004; Ohler *et al.* 2009) which may, in extreme cases such as the smaller species of the Madagascar genus *Stumpfia*, result in the loss of digits (Glaw & Vences 2007). Another factor that limits the interpretation of morphological characters more generally in frogs is the widespread occurrence of convergent evolution and remarkable similarity in overall morphological features (Bossuyt & Milinkovitch 2000).

In addition, substantial variation between individuals was obtained in this study. This hindered the use of these characters in the construction of phylogenetic trees.

The x-ray images taken in this study were sensitive to the angle of the limbs of the specimen although almost all specimens were fixed in a standard manner to facilitate morphological comparison. However, there were still some angular effects due to different degrees of spinal flexion and as a result I do not have a high degree of confidence in these observations. The use of 3D microtomography scans may be required to assess finer skeletal characters.

The few significant and highly significant differences between some species, even with these limited sample sizes and other caveats, warrants further investigation of the utility of morphological characters. The geometrical morphometric approach of Alibert *et al.* (2001) may be a useful method to compare skeletal characters using morphological landmarks.

4.4 Phylogeographic patterns

The spatially clumped distribution of most *Arthroleptella* populations follows the patchy distribution of suitable (permanently moist) habitat. Suitable habitat will have become more patchy over time as the climate of the Cape has changed from a mesic climate to a climate with very dry and hot summers. This restricts the number of environmental locations where moisture levels remain high throughout the year. Moss frogs have not evolved to tolerate dry environments as have some other members of the Pyxicephalidae in the Western Cape such as *Tomopterna delalandii* which can burrow through dry soil to aestivate during the austral summers. The steep climatic gradients in the CFR are exacerbated by edaphic diversity (Cowling *et al.* 1992; Goldblatt & Manning 2002; Cowling *et al*. 2009). Edaphic specialists will experience severe reductions and may loose intermediate populations in the presence of fire (short term) or climate change (long term)

(Cowling *et al.* 1992). Cowling *et al.* (2009) further emphasise the role of edaphic diversity in driving speciation of the CFR plants. Given the clear patterns of association between *Arthroleptella* and geology and soils it is likely that edaphic patterns have influenced the distribution and speciation patterns in *Arthroleptella*.

The pattern of population-level differences in *Arthroleptella* gene sequences and calls, even over fine spatial scales may also be a result of their mode of reproduction as terrestrial direct developers. Vences & Wake (2007: p2617) suggest that "within direct-developing species in particular, differentiation in situ most probably proceeds through a combination of neutral and selective factors operating over long spans of time". These authors suggest that these factors in conjunction with spatial heterogeneity may have lead to the abundant speciation that has taken place in relatively small areas in other direct developers such as *Thorius, Eleutherodactylus* and *Philautus*. That *Arthroleptella* species have small clutch sizes unlike *Philautus* (Meegaskumbura *et al.* 2002) may have further enhanced the selection pressures to produce divergent populations over fine spatial scales.

The large degree of variation of advertisement calls across space was an unexpected result as it is counter to the conservation of mate recognition signals which is predicted by the Recognition Concept (Paterson 1985). The cases in which there was a decoupling between genetic relatedness and advertisement call similarity are instructive. They indicate that advertisement calls may drift with isolation but may also be maintained despite isolation. These effects can occur over small spatial scales so the spatial degree of spatial isolation is unlikely to be the factor responsible for the different patterns. Other factors such as historical minimum population sizes may well be involved but I do not yet have sufficient population-level genetic data to investigate this hypothesis. The role of fire in both fragmenting and reducing population sizes is still unknown but is likely to have been a very important driver of population processes in *Arthroleptella* over the past nine to five million years since the initiation of a fire-regime in the CFR. This will have been particularly acute over the past three million years as the Benguela upwelling intensified to present levels causing summer aridity and hence increasing the probability of fires. In plants, fire-induced mortality increases generation turnover, thereby providing potential for more rapid evolution in re-seeders versus re-sprouters after fires (Wisheu *et al.* 2000). An analogous effect is suggested to operate on *Arthroleptella.* They are fire-prone which will cause rapid turn-over of local populations. This in conjunction with their poor dispersal abilities will lead to populations becoming isolated and diverging on separate evolutionary pathways.

The general phylogeographic pattern of differences between each mountain range in the CFR displayed by the genus is not clearly shown by *A. villiersi*. In this taxon the lack of a clear geographical pattern in the genetic sequences indicates that different evolutionary processes have operated. This is corroborated by this taxon's wide distribution and tolerance of habitats that would be unsuitable for other species of *Arthroleptella*. This lack of genetic structure in conjunction with the DIVA results, suggests that *A. villiersi* is capable of much longer distance dispersal than its congeners. The polyphyly in the phylogenetic trees and reticulate patterns in the network reconstructions in the *A. lightfooti* and *A. villiersi* clade indicates that there may have been a complicated history of colonisation, local extinction and recolonisation across the range of this clade. Such processes have been suggested to complicate, or even preclude, the accurate recovery of the history of species (Chek *et al.* 2001). Further investigation of the dispersal ability and habitat requirements of *A. villiersi* may yield an improved understanding of their unusual current distribution.

4.5 Dispersal

Variation in genetic sequences over geographic distance in most species of *Arthroleptella* indicates that there is little gene flow between populations (as shown by Mantel tests, AMOVA, SAMOVA and pairwise genetic comparisons) indicating limited dispersal between populations. This result is supported by several factors outlined below.

Arthroleptella are very small frogs and hence have a very large surface area to volume ratio. This makes them vulnerable to dehydration. They are habitat specific and require permanent moisture and dense cover. They are secretive frogs and generally do not expose themselves at surface level, preferring to remain hidden within the vegetation. Their normal mode of locomotion is a slow crawling that allows them to move through the dense vegetation and decaying organic matter in which they live. They are expected, like many other frogs, to display philopatric breeding (Beebee 2005), further reducing the potential for gene flow between populations.

Appropriate habitat is sparsely and patchily distributed in the CFM requiring large dispersal distances to reach the next patch of suitable habitat. This may be mediated by being able to travel along drainage lines where adjacent vegetation may be moist enough to allow linear dispersal. However, few species utilise vegetation adjacent to streams or rivers except at the highest reaches where the streams are still part of seeps and generally avoid areas with flowing water. The geographic patterns of genetic disjunctions are often associated with rivers indicating that when it comes to crossing rivers, rivers are significant biogeographic obstacles to *Arthroleptella*. Rivers

have been shown to constitute significant barriers to gene flow and have contributed to speciation in the genus *Pseudacris* (Lemmon *et al.* 2007). The exceptionally widely distributed *A. villiersi* has been recorded to move a distance of approximately 75 m over a wet season along a drainage line near a seep when adults were heard calling at a new locality near the Swartboskloof CapeNature long-term frog monitoring site - a site that has been regularly monitored over the last 7 years. A very small population of *A. landdrosia* present at one of the Landdroskop long-term frog monitoring sites was last observed in 2002 and has not been recorded there since, despite a population present only 250 m (straight line distance, 350 m along drainage lines) away. It is unlikely that the other species of *Arthroleptella* are capable of dispersing much more than this distance in a year. Observations of permanently marked animals would be most useful in establishing maximum dispersal distance in *Arthroleptella*. Alternatively, population level genetic analyses in an area where potential dispersal corridors exist may also yield useful indications of dispersal ability.

In summary, the small size and dependence on permanent moisture in *Arthroleptella* makes long-distance dispersal difficult in this genus. This is because for most of the year the inter-habitat matrix is completely dry and even immediately after rain, moss frogs would have to cover very large distances between suitable places that provide sufficient shelter and moisture. The rapid run-off of water on the slopes of the mountains leaves only small seeps and streams as wet corridors for dispersal and means that the effects of rain are short lived. Therefore long-distance dispersal is only possible under conditions of continuous or very regular rainfall events. Although such events must occur within long time periods, the relative rarity of these events reduces the probability of successful migration for moss frogs. It is likely that similar processes are operating in the genus *Capensibufo* which has recently been shown to display a phylogeographic pattern that closely mirrors the findings of this study with distinct genetic disjunctions between different mountains of the CFM (Tolley *et al.* 2010b). A similar pattern has been found for several montane rainforest frogs (Carnaval 2002; Bossuyt *et al.* 2004).

Arthroleptella villiersi: the exception

One species, *A. villiersi* is patchily distributed but very widespread in contrast to all other species. This indicates that this species is capable of longer distance dispersal or can survive in a greater variety of habitats or has had a different evolutionary history to the other species. These explanations are not mutually exclusive.

Firstly, there is evidence that *A. villiersi* is less fussy about the habitat in which it occurs and breeds. I have found this species calling in fynbos during very wet weather that would at other times be totally dry and devoid of any *Arthroleptella*. Other species of *Arthroleptella* do not call from vegetation that is not permanently moist. *Arthroleptella villiersi* has also been found calling in pine plantations which are artificial habitats that are very different to the habitats in which this species is naturally found. Despite this versatility in choice of habitat they are still restricted to moist habitats at any given time. They, like all the other species, have not been found on clay substrates. Secondly, *A. villiersi* has proportionately the longest legs of all species in the genus (Table 62). Longer legs have been related to greater dispersal abilities in other frogs e.g. *Rhinella marina* (Phillips *et al.* 2006), and jumping performance *Pelophylax lessonae-ridibunda* hybrids e.g. (Tejedo *et al.* 2000; Choi *et al.* 2003) and a number of other frog species (Choi *et al.* 2003). This evidence supports the inference that *A. villiersi* has a greater probability of long-distance dispersal due its ability to move greater distances with its longer legs and its tendency to use new habitats if they are moist enough (even if only for a short time). However, long-term survival of populations will only occur in places of permanent moisture availability. In contrast, the other species are very restricted to their breeding habitats and are inferred to be poor at dispersal. That *A. drewesii* also has relatively long legs indicates that additional factors are involved in the dispersal ability of *A. villiersi* as *A. drewesii* has a very limited range.

Another possible explanation for the current wide distribution of A. *villiersi* is that it was originally more uniformly distributed over a large range and that the current range is now composed of vicariantly fragmented populations. If this were the case, then one would expect significant genetic differentiation amongst these populations. Although genetic variation within the species is not low in comparison the other species, geographically distant populations of *A. villiersi* show remarkably little genetic divergence *viz*. populations from the extreme north-west (Paarl Mountain) and south-east (near Cape Agulhas) had identical 16S sequences. This geographically scattered pattern of genetic diversity is more likely to be the result of dispersal rather than vicariant processes (Phillips 1994) and is supported by the DIVA analysis which indicated 5 dispersal events for *A. villiersi*. I consider the interpretations derived from the DIVA analysis to be robust as they are consistent with a relatively simple pattern of repeated vicariant events indicated by the other phylogeographic evidence presented in this study (*cf.* Kodandaramaiah 2010). The interpretation of dispersal is supported by the widespread distribution of *A. villiersi* in strong contrast to the restricted distributions of the other *Arthroleptella* species. Again this supports the general interpretation that *Arthroleptella* are habitat-restricted and population divergence is due to vicariance with the single exception of *A. villiersi* which seems capable of longer distance dispersal.

Maximum intraspecifc variation as measured by uncorrected p-distance is relatively high in *A. lightfooti* compared to *A. villiersi* when the sizes of the distributions are taken into consideration *viz*. 2.0 % over a linear extent of 33 km vs. 4.0 % over 145 km respectively. This result should be investigated further by finer spatial sampling of both taxa.

The *A. villiersi* population on Paarl Mountain deserves further consideration as it is an outlier in several ways. Firstly, Paarl Mountain is a granite extrusion rather than part of the CFM. Secondly, only the very summit of the mountain supports fynbos vegetation. The mountain slopes and surrounds are covered in renosterveld which is unsuitable for moss frogs. The lack of significant genetic divergence of this population from other *A. villiersi* populations indicates that their presence on this mountain is a relatively recent dispersal event. The results of the DIVA indicate that *A. villiersi* has dispersed to occupy the large current range.

4.6 Fire and its effects on population dynamics and speciation

In the CFR, fire has been suggested to contribute to increased environmental heterogeneity and as a direct driver of speciation among plants (Cowling 1987; Cowling *et al.* 1992; Van Wilgen *et al.* 1992; Linder & Hardy 2004). This is more obvious in the plants of which many show clear adaptations to surviving fire. The pressure exerted on the CFR plants to develop fire-tolerant strategies is argued to have offered a competitive advantage to Cape flora overt the pre-existing tropical floras (Linder & Hardy 2004). As the vegetation changed from more tropical elements to the modern fynbos, moss frogs too would have had to shift their ancestral habitat choice from more wooded vegetation to permanently wet places in the newly emerging fynbos. Fire also temporarily destroys habitat and may threaten frogs directly. Post-fire population extinctions in plants are not uncommon (Cowling 1987). The patchy nature of fires serves to further fragment habitat and frog populations. The pattern of fires over time will affect the distribution pattern of frogs as populations are split and destroyed and as suitable habitat patches form and are colonised. This process is likely to create a more complex pattern of distribution on the landscape as this process overlies the spatial patterns created by dispersal and other population level processes. Additionally, habitat specialists are more susceptible to directional selection in the event of environmental change leading to fragmentation, population divergence, vicariant speciation and extinction (Cowling *et al.* 1992)

Fire is expected to affect moss frogs by increasing barriers to gene flow by removing vegetation, decreasing soil moisture and reducing population sizes and so enhancing potential for local population divergences. Fires do not burn or do not burn well in permanently wet places and this

places further strictures on habitat choice for moss frogs. This habitat choice effect will work in concert with their low desiccation tolerance. It will be challenging to tease apart these two separate ecological selection pressures as they will tend to result in the same behaviours and distribution patterns.

I consider fire to be a very important determinant of suitable habitat and population dynamics in *Arthroleptella* as all species are highly dependent on permanent moisture and dense vegetative cover. The limited observations made on post-fire presence support the view that fire does influence local population dynamics by causing severe population size reductions and local extinctions. This will have the effects of reducing effective population size and increasing the spatial fragmentation of metapopulations. Both these effects are likely to enhance possibilities for the development of divergent gene lineages due to the reduced gene flow between smaller, more fragmented populations (Hanski 1999). This may result in populations that previously functioned as metapopulations becoming completely isolated. Frog populations are frequently regarded as metapopulations, briefly reviewed by Alford & Richards (1999). This work and the work of others such as Driscoll (1998) indicates that frog populations, even in close geographic proximity, may not form part of a metapopulation. The combination of fire driving habitat and directly affecting population dynamics is argued to have played an important role in the diversification of and eventual speciation in *Arthroleptella*.

4.7 Distribution & habitat associations

The clear association of *Arthroleptella* with TMS and their absence on shale and shale-derived soils can be explained by the water retention properties of these two geological types and the soils derived from them. Shales and shale-derived clays have very high moisture retention abilities due to their fine particle size and physical properties of their chemical composition (see Lechmere-Oertel & Cowling (2001) for the effects on fynbos vegetation) such that in the dry summer months there is no surface water either directly available to the frogs (Hillman *et al.* 2009) or indirectly to the mosses and fungi in which moss frogs shelter. The quartz sands derived from TMS are relatively coarse and do not absorb water to the extent that clays do and are thus easily saturated. Previously, Van Dijk (1982) noted an association of the brevicipitid *Breviceps montanus* with TMS soils but did not investigate soil associations with *Arthroleptella*. It is noteworthy that the older elements in fynbos are largely endemic to the sandstones of the Cape Fold Mountains (Verboom *et al.* 2009).

The presence of moss frogs on TMS and granite is further restricted to seep areas and other permanently moist areas. Both TMS and granite drain water very effectively and much of this

substrate is dry in the summer months. Thus there is an interplay between geology and topography such that the frogs are only able to survive in areas where the topography is such that water accumulates year round (either from subsurface flows or precipitation, particularly that supplied by mist and fog) and where the soils or rock make the surface water available to the frogs.

The water available to frogs is also influenced by the amount of organic matter on the surface and in the uppermost soil horizons in which the frogs live. Moss frogs are always found in dense vegetation so there is always surface organic matter. As these areas are generally permanently wet they are less prone to fire and organic matter tends to accumulate in larger seeps and may form peat. In general, high organic matter content improves moisture availability and contributes to a site's suitability as potential habitat for *Arthroleptella* species.

Geomorphological evolution has largely been ignored as a driver of the evolution of the unique suite of species of the CFM (Cowling *et al.* 2009). Moss frogs have also been affected by geomorphological evolution. Suitable habitat for *Arthroleptella* is largely determined by a conjunction of suitable soils (derived from TMS) with landscape position that allows accumulation of surface moisture but not fast, free flowing water. The evolution of unsuitable habitat (e.g. fast-draining rocky slopes) will have strengthened the barriers to gene flow between populations and has thus been a significant force in shaping population evolution and speciation in moss frogs. This thesis presents evidence that the influence of geomorphological evolution is not confined to the CFM flora (Cowling *et al.* 2009) but also applies to a group of small, endemic and habitat-specific vertebrates.

The predictable pattern of rainfall in the mountains of the CFR is argued to be one of the factors that allowed for the persistence of *Arthroleptella* in a novel and physiologically challenging environment for a very small anuran. Stable environments such as these are conducive to speciation as they promote the accumulation of different genomes (Fjeldså & Lovett 1997; Cowling & Lombard 2002). However, in the case of *Arthroleptella* the stable regions are fragmented, small and rare across the CFR. This will have promoted the evolution of distinct genetic lineages and is likely to have been the primary evolutionary driver of diversity and speciation in *Arthroleptella.*

4.8 Conservation

4.8.1 Assessing threat status

The primary data available for assessing threat status is distribution data. Although this data is useful it only provides a rough quantification of threat status. However, now that relatively good

distribution and habitat maps have been constructed, future habitat loss should be quantifiable and will inform Criterion A (changes in population size). Detailed monitoring of a sample of populations can be used for estimations of population numbers and will provide insights into population dynamics over time which are required for assessing criteria A, B and C. Information is also required on generation time. Once all these population descriptors have been collected, population models can be parameterised to model extinction risk which can be used in criterion E.

4.8.2 Invasive alien plants as a threat

Alien invasive plant species are a striking and even dominant feature of many fynbos landscapes (Richardson *et al.* 1992). The effects of alien invasive plants on hydrological regimes in the Western Cape Province are well documented and have been shown to decrease total runoff and surface moisture through their increased evapotranspiration rates (Le Maitre *et al.* 2002). They also often form very dense stands which competitively exclude indigenous vegetation (Van Wilgen & Richardson 1985). The negative effects that these species have on habitat for *Arthroleptella* and other moisture-dependent species is exacerbated by their contribution to fuel load for fires (Van Wilgen & Richardson 1985). This results in more intense fires which will further desiccate habitat. Alien invasive tree species such as *Pinus pinaster* and *Hakea sericea* are present on most of the mountain ranges on which *Arthroleptella* species occur. The severity of the infestation varies considerably and hence the threat to the survival of the *Arthroleptella* species varies concomitantly.

There is an unquantified threat posed by water abstraction. The TMG is highly fractured and functions as an aquifer (Roets *et al.* 2008b). The water retained in the TMG has been identified as a resource for human use (see http://www.tmg-aquifer.co.za). The effects of groundwater extraction on habitat for *Arthroleptella* depend on the particular part of the aquifer that will be utilised. If the water is extracted from the 'TMG aquifer daylight-domain' which is in located in the recharge zone (see Figure 76), the lower flows of the flow regime will be affected most. Roets *et al.* (2008a) note that it is this domain that will be most vulnerable to groundwater use. *Arthroleptella* habitat is located on TMG mountain slopes within this domain. The entire distribution of *Arthroleptella* is included in the area identified as sensitive aquatic ecosystems vulnerable to groundwater use from the TMG (Roets *et al.* 2008a).

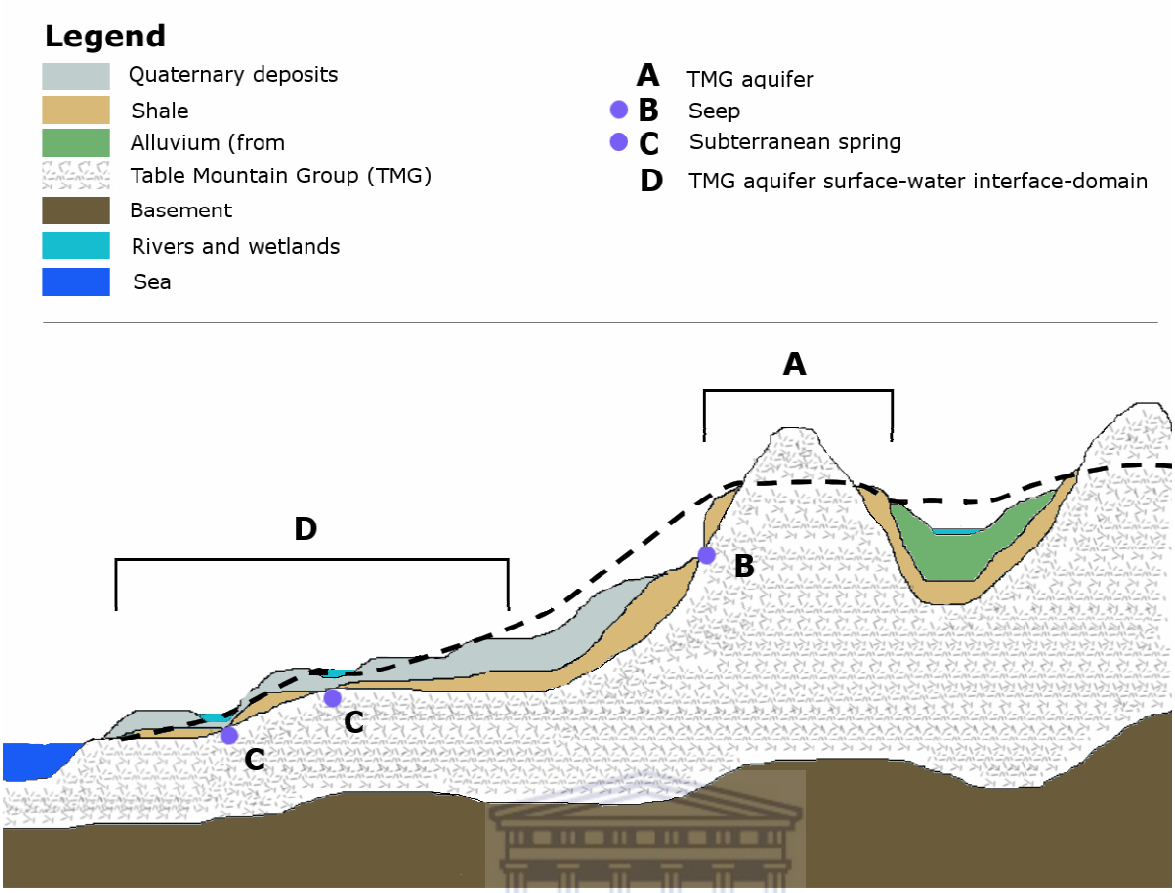

Figure 76. Diagram showing the location of the TMG aquifer daylight-domain (from Roets *et al*. 2008a).

4.8.3 Managing threats

Fire

Vulnerability of a habitat to fire is difficult to measure but observations in the field indicate that seeps in open exposed areas must be very well developed i.e. very wet throughout the year or close to rocky outcrops.

Wilson *et al.* (2010) found that fire occurrence in the CFR is strongly affected by climatic variability at both local and global scales, and thus likely to respond sensitively to future climate change which they predict will result in a substantial increase in fire frequency. This will have a direct negative impact on *Arthroleptella* species. It remains to be seen whether they will be able to respond to the rapid onset of an increased frequency fire regime. This is an area worthy of further research and development of mitigation measures to maintain suitable habitat in the face of an increased number of fires. This can only be achieved by active fire management in which permanently moist areas such as mountain seeps should be afforded priority protection in fires that are occurring too frequently. The definition of 'too frequent' is a frequency above the threshold

(threshold of potential concern *sensu* Biggs & Rogers (2003) determined for the fynbos vegetation of a particular area. The determination of these thresholds is an ongoing process within CapeNature. This does not mean that fires should never be allowed to burn through seeps, only that such a burn should be a rare occurrence and for most of the moist south-western parts of the Western Cape should be at a fire return interval of no less than 20 years. This figure is based on the time required for sufficient vegetative recovery in this area rather than on the time required for an *Arthroleptella* population to reach saturation levels (i.e. reach carrying capacity).

The long-term survival of moss frogs in the fire-prone fynbos indicates that they are capable of persisting under natural fire regimes. This is likely to be due to the survival of a small number of individuals in well-developed seeps where burns do not consume 100 % of the organic material in combination. In cases where seeps are close enough for dispersal to be successful, local metapopulation dynamics may be capable of rescuing local extinctions. Although I have not explicitly tested the requirements for considering *Arthroleptella* populations as metapopulations (see Smith & Green 2005 for a review of evidence for amphibian metapopulations), the data on distribution, fire responses and genetic structuring indicate that *Arthroleptella* populations function as metapopulations.

The invasion of fynbos habitats by invasive alien plants dramatically increases fuel loads and thus the heat generated by fires in invaded areas, particularly when such trees have been felled (van Wilgen *et al.* 1992). Fires under these circumstances are predicted to have a greater effect on population reductions of moss frogs and habitat damage than fires in uninvaded areas.

The increasing frequency of fires in the fynbos (Van Wilgen & Forsyth 2008) in conjunction with the continued presence and expansion of alien invasive plants means that *Arthroleptella* populations will be at greater risk of local extinctions, and ultimately species extinction, unless these threats are mitigated or halted. This is of particular concern for those species with highly restricted distribution ranges such as *A. rugosa*.

Invasive alien plants

Invasive alien plant species are a serious threat to many parts of the CFR and act in concert with fires to cause more severe damage to habitats. As the fynbos is a fire-prone area and is extensively invaded by alien invasive plant species, these threats are continually present. Active management i.e. removal of invasive alien plants and management for an acceptable fire-return interval, particularly in seeps is required to keep these threats in check.

Global climate change

The genus *Arthroleptella* is restricted to cold and wet environments and currently occupies a relictual distribution characterised by small species ranges. Centres of high richness of small-range species have been found to correspond with interglacial refugia (Ohlemüller *et al.* 2008). They show that these species will be disproportionately negatively affected by future climate changes. A model of global climate change predicted greater changes (species turnover) in local amphibian fauna than in either mammal or bird fauna (Lawler *et al.* 2009). Additionally these authors found that many of the areas predicted to experience large faunal changes are in mountainous regions where environmental conditions vary significantly over relatively short distances. These conditions apply to *Arthroleptella*.

Locally, climate change has also been predicted to have negative consequences on reptile species genetic diversity in the CFR (Tolley *et al.* 2009). An important long-term conservation question is whether the historical pattern of the western CFR as a habitat (great niche diversity) and weather refuge (continued precipitation in spite of global climate shifts) will pertain with anthropogenic climate change? It is unlikely due to the vast number of anthropogenic barriers to gene flow in *Arthroleptella* e.g. roads, agricultural lands and urban areas (see Hitchings & Beebee (1997); Davis & Shaw (2001) for a review of these effects on frogs and on trees which have much greater dispersal abilities). This means that only the best placed populations of *Arthroleptella* relative to permanent moisture and good ground cover will survive. In addition, these populations will not be able to pass on local adaptations to other populations even if there were sufficient time for these to evolve in the short time span over which anthropogenic climate change is occurring. However, the persistence of these populations is dependent on microhabitat persistence rather than general climatic conditions. If the microhabitats persist then the frogs are also expected to persist in the face of climate change as they have done in the past up to the point of their physiological thermal tolerance. Anecdotal observations made on captive individuals indicate that they become stressed at temperatures above 30º C and may die at 37º C.

An indirect effect of climate change that is predicted to be more immediate is the facilitation of growth and spread of invasive alien woody plant species which will directly affect moss frogs in addition to exacerbating the deleterious impacts effects of fire.

Protected areas

There may be more wide-ranging implications of the finding in this study of greater diversity within the genus *Arthroleptella* than previously known. The pattern of little or no gene flow between the various massifs of the CFM may apply to other animals that have not yet been studied. It is already well-known for plants and may raise the conservation importance of even relatively small areas if they support multiple unique genetic lineages (see Kahindo *et al.* 2007 for an afro-montane bird example). The high levels of genetic divergence across *Arthroleptella* populations is typical of the distribution of diversity across fynbos plants and means that a comprehensive network of protected areas is required to adequately represent the amphibian and other biodiversity of this region. Fortunately many of the mountainous regions of the CFR fall into the existing national and provincial conservation network. A notable exception is the Klein Swartberg Mountain near Caledon which is the sole locality for *A. rugosa*.

4.9 Conclusion

The genus *Arthroleptella* has a relictual distribution with ten species that occupy small and localised habitats and with a single species displaying an ability to disperse widely across the fynbos landscape. The historical move from forest dwelling ancestors to the more open habitat of the fynbos is a response to changing climate and vegetation.

There is a general pattern of substantial genetic and call variation over small geographic distances. This is consistent with the interpretation that these frogs have strong habitat preferences and low vagility.

Reconstructing ancestral relationships and geographic relationships relies heavily on genetic data but there are several other lines of evidence that can be combined to give greater insight into past evolution. In this study, advertisement calls contributed to an understanding of patterns of population evolution and speciation. Biogeographic evidence in the form of current and past habitat distributions, as inferred from climatic and geological evidence, has been used to construct a model of shrinking habitat with the drying and cooling of the South Western CFR. This habitat is overlain on a complex geological and topographical landscape in which barriers to dispersal (rivers and clay soils) have generally prevented dispersal and increased fragmentation and isolation of populations of moss frogs. This approach in which evidence from several different disciplines is combined has been shown to benefit the reconstruction of historical biogeography (Phillips 1994) and contributes to a more complete model of the evolution of the genus *Arthroleptella*.

Cowling (1987) noted that patterns of plant diversity in the CFR are not unlike those in the species-rich tropical rainforests. Southern Africa has been considered a 'cradle of diversity' due to the many recently-evolved species (Tolley *et al.* 2008). The relictual pattern displayed by *Arthroleptella* indicates that the some of the faunal diversity of the CFR is due to persistence of isolated lineages over long periods of time and that the CFR may be also be considered a 'museum of diversity'. This extends to several other relictual taxa such as birds in the genera *Chaetops* and *Promerops* (Fjeldså & Bowie 2008) and heleophrynid and pipid frogs. The concepts of 'cradles' and 'museums' of diversity are not mutually exclusive (Chown & Gaston 2000). The ability to date lineage formation now provides clearer insights into the processes that generate the terminal taxa and hence species diversity and should help distinguish 'cradles' from 'museums' although there may be many areas in which both of these processes operate.

4.10 Future research

There are still a few sections of the Cape Fold Mountains that have not yet been surveyed for the presence of moss frogs and genetic samples from these may yield new insights into the relationships between the populations used in this study. In species that appear recently evolved such as *A. lightfooti* and *A. subvoce* a finer phylogeny using more variable gene fragments may lead to a better understanding of the evolution of these populations. In addition there is already substantial evidence for fine geographic scale genetic patterning within *A. landdrosia* which may be obscuring further cryptic or incipient species. Questions that should be asked in this context are 'why is the Eerste River such a barrier?' and 'is the Houwhoek population an incipient species or is it a functional species already?' I suggest that a suite of gene fragments such as Mt ND1, ND2 or ND4 + beta-fibrinogen intron 7 be used to examine finer scale patterns. Also a more variable nuclear exon will help basal resolution of the genus. The difficulties in amplifying the ND2 gene may be resolved by obtaining complete mitochondrial sequences for representative members of *Arthroleptella* to check for gene rearrangements (Sano *et al.* 2004) or unusual sequences in the primer regions.

The variability in calls requires data from more individuals to better assess variation between populations, individuals in a population and within individuals. Social interactions between individuals should be investigated as a possible explanation of intra-individual variation. This could be achieved by call playbacks to males. However, observation of such behaviour may be difficult and techniques that make use of aural responses or the use of very small remote video camera lenses may be required.

The ability of *A. villiersi* to disperse, breed, and better survive fires in contrast to its congeners deserves further investigation. Comparative physiological studies may shed light on this interesting phenomenon.

The current data available with which to assess threat status, as required by the IUCN, is only of reasonable quality regarding the extent of occurrence of *Arthroleptella* species. To enable more accurate threat assessments, better data are required on area of occupancy and quantitative measures of habitat requirements, population size, movements between populations and the effects of fire and invasive alien vegetation. Still to be determined is the total reproductive output per female as it is not known how many clutches can be laid per year. The mapping of fine scale habitat should be possible in the near future through improved remote sensing data classification techniques and increasingly fine spatial scale remotely sensed data. An estimation of the size of *A. subvoce* should be feasible as one population is regularly monitored and the other two known populations can be reached by a one day hike. It will be instructive to know how long the various species take to reach maturity and what the longevity of individuals is. It will also be important to quantify the actual reproductive output of individuals. These are difficult things to measure in the field, (maintenance in captivity is even rather challenging) but quantification may perhaps be possible under semi-natural conditions using in-situ enclosures.

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Appendices

Appendix 1. Species accounts

Arthroleptella lightfooti (Boulenger 1910)

Lightfoot's moss frog, Peninsula moss frog

Photographs

Figure 77. *Arthroleptella lightfooti* male from Olifantsbos, Table Mountain National Park.

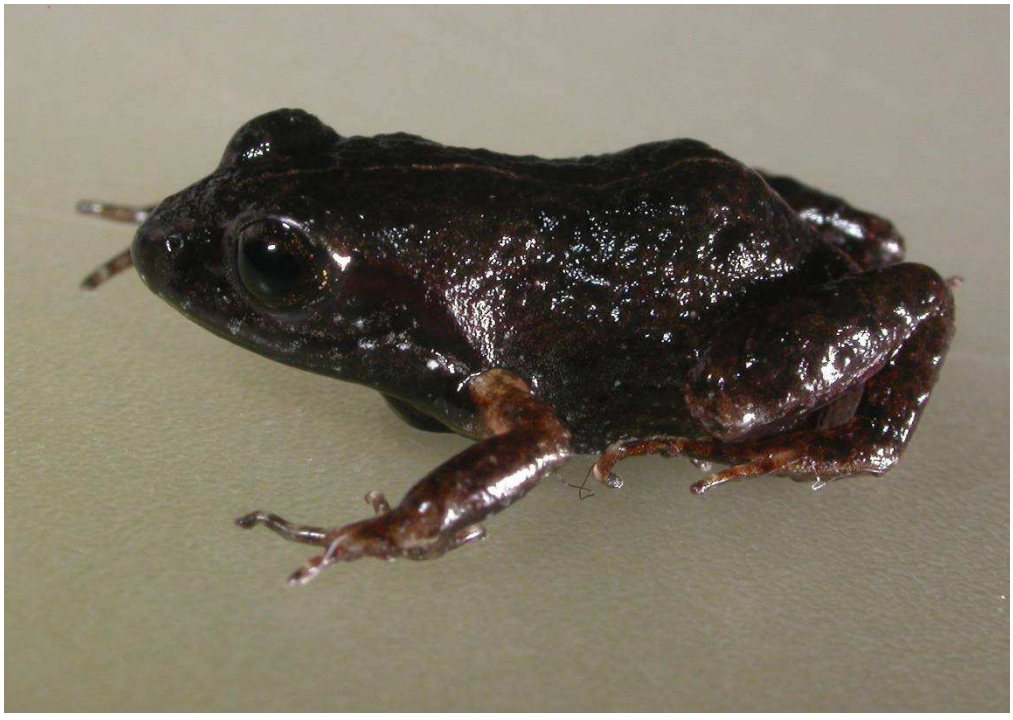

Figure 78. *Arthroleptella lightfooti* male from Silvermine, Table Mountain National Park.

Figure 79. *Arthroleptella lightfooti* male from the Cecilia plantation, Table Mountain National Park.

Figure 80. *Arthroleptella lightfooti* male from the Back Table, Table Mountain National Park.

Call

The call is a melodious, bell-like chirp. The melodious character of the call is difficult to quantify but it stands in contrast to the harsher call of *A. villiersi*. The calls may be in groups of two or occasionally three. The acoustic measurements of calls of *A. lightfooti* and *A. villiersi* are compared

in Tables 7 to 20. In summary, the two calls can be differentiated as follows: *A. lightfooti* has fewer pulses per note (3.6 vs. 4.2) and generally a lower dominant frequency (3794 kHz vs. 3802 kHz).

Appearance

The dorsal skin is granular with some raised bumps in males; the skin is smooth in females. The toe tips are slightly expanded. The finger tips are not expanded. Colouration is very variable in this species (see photographs above). Colour is most often a relatively uniform dark orange-brown but may be purplish-brown to black above. Markings are generally obscure and often consist of irregular dark marks over the upper thighs and a dark streak from the eye to the insertion of the arm. Adult females are white below. Breeding males have a black throat, including laterally folded vocal sac and the black colour usually extends onto the belly.

Distribution

Lightfoot's moss frog is restricted the Cape Peninsula from Cape Point to Table Mountain. Much of its distribution falls within the Table Mountain National Park.

Habitat

Seepage areas along side streams and on wet plateauxs, primarily in fynbos but also marginally in afromontane forest.

Breeding

Calling starts in autumn in April and may extend to November but rainy weather may prolong the calling period. Clutch size varies from 5 to 12 eggs (Channing 2001).

Systematics

A. lightfooti is the type species for the genus *Arthroleptella* (Hewitt 1926) and was the only recognized species in this genus in the Western Cape for most of the 20th century. The systematics of *A. lightfooti* have thus been confused with most of the Western Cape species with the exception of *A. rugosa* which was unknown till very recently. The restricted and allopatric distribution of *A. lightfooti* makes retrospective assignment based on geographic location possible. Specimens of uncertain geographic provenance cannot be reliably ascribed to *A. lightfooti* on morphological traits alone as it is very similar to its sister species *A. villiersi*. The calls of these two species are very similar when analysed by standard call parameters. The calls show acoustic differences although these overlap to some extent.

***Arthroleptella villiersi* (Hewitt 1935)**

De Villiers' moss frog, chirping moss frog

Photographs

Figure 81. *Arthroleptella villiersi* male from Babilonstoring Mountain.

Figure 82. *Arthroleptella villiersi* male from Landdroskop, Hottentots-Holland Mountains.

Figure 83. *Arthroleptella villiersi* female from Paarl Mountain.

Appearance

De Villiers' moss frog is relatively longer-limbed and sharper-snouted than all other species. *Arthroleptella villiersi* is usually a khaki colour with lighter dorsolateral bands and frequently a paler snout. Colour varies from yellowish brown to greyish brown. Males are almost entirely black below with some paler mottling towards the posterior regions. The colour patterns shown in Figures 68 and 69 are commonly encountered. Females are immaculate white below.

Call

The call is a short chirp that is frequently uttered in groups of two and occasionally in groups of three. The call has a harsh quality and is generally higher pitched than the call of *A. lightfooti* although the calls are similar and other call measurements do not differ significantly between *A. villiersi* and *A. lightfooti*.

Distribution

Arthroleptella villiersi has a wide distribution from Paarl Mountain and the Franschhoek Pass in the north extending southward along the Hottentots-Holland Mountains to the coast from the Kogelberg Mountains and then eastwards to just west of Cape Agulhas on the coast and inland just east of Napier.

Habitat

During the rainy season this species may be found in a variety of damp areas from stream-side seeps, mountain seeps and bogs and flat places where water can accumulate, even temporarily, in the soil.

Breeding

Calling extends from at least April to December. Clutch sizes vary from 4 (M.J. Cunningham & C.L. Henderson unpublished data) to 11 (Morgan *et al.* 1989).

***Arthroleptella rugosa* Turner & Channing 2008**

Rough moss frog

Photographs

Figure 84. *Arthroleptella rugosa* male from Klein Swartberg Mountain near Caledon.

Figure 85. *Arthroleptella rugosa* female from Klein Swartberg Mountain near Caledon.

Appearance

The dorsal skin of both males and females is covered in numerous raised bumps. The snout is rounded. The limbs are relatively short. The dorsum is dark chocolate brown, occasionally with a lighter brown on the dorsolateral surfaces. The underside is black over the throat and chest region and speckled dark grey over the remainder in males. Females are uniformly dark grey below speckled with white.

Call

Unique among *Arthroleptella*, the rough moss frog has two types of advertisement call: the first is a rough, squeaky sounding chirp and the second is a longer 'chuckle' call (see Turner & Channing 2008). Both calls are easily distinguished from all other *Arthroleptella* in the field.

Distribution

Restricted to the southern slopes of the Klein Swartberg Mountain near Caledon.

Habitat

Well developed mountain seeps with tall restioid vegetation.

Breeding

Calling has been recorded from May to December. Three clutches have been recorded with 7, 8 and 6 eggs.

***Arthroleptella bicolor* (Hewitt 1926)**

Bainskloof moss frog

Photographs

Figure 86. *Arthroleptella bicolor* male from the Bainskloof Pass, Limietberg Nature Reserve.

Figure 87. *Arthroleptella bicolor* male from the Bainskloof Pass, Limietberg Nature Reserve showing unusual yellow colouring (this specimen was even brighter yellow upon capture).

Figure 88. *Arthroleptella bicolor* male from the slopes of Observation Peak, Limietberg Nature Reserve.

Appearance

The Bainskloof moss frog has a squat appearance with a rounded snout. Finger and toe tips are very slightly expanded. Colour is variable in shades of brown to grey-brown and yellow-brown but is

often a dark reddish brown with irregular dark mottles over most of the dorsal surfaces. The throats of males are black. Females are clear white below.

Call

A highly irregular set of click notes.

Distribution

It is distributed on the Limietberg and Waterval Mountains.

Habitat

Well developed mountain seeps in fynbos.

Breeding

Channing (2001) reports eight to ten eggs in a clutch but it is not clear whether these data included *A. sp.* A which is distinct from *A. bicolor*.

***Arthroleptella subvoce* Turner, De Villiers, Dawood & Channing 2004**

Northern moss frog

Photographs

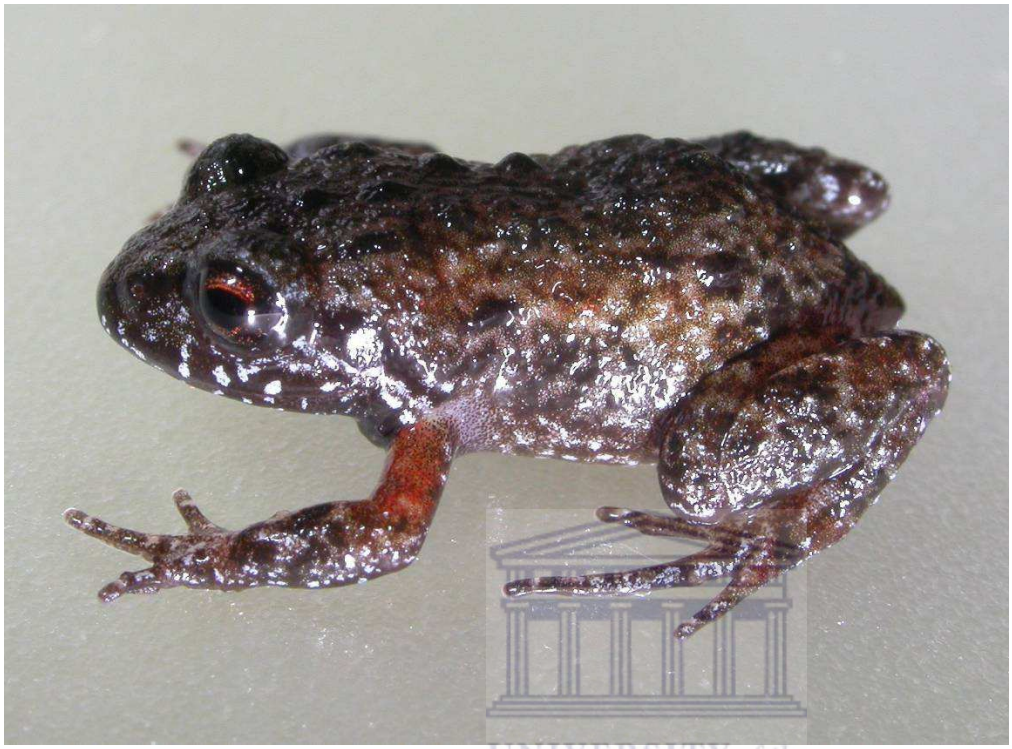

Figure 89. *Arthroleptella subvoce* male from the Grootwinterhoek Wilderness Area.

Figure 90. *Arthroleptella subvoce* female from the Grootwinterhoek Wilderness Area.

Appearance

This species is probably the smallest *Arthroleptella* species with adult males reaching a snout-urostyle length of 14 mm. The snout is very rounded and the finger and toe tips are not expanded. Colour is mostly brown with irregular darker brown patterns dorsally. The dorsal surface of the upper arms is orange-brown. Males have a grey throat and are pale grey speckled with darker grey over the rest of the underside. The vocal sac is poorly developed. Females are speckled grey below.

Call

The call is a chirp followed by a number of clicks (Turner *et al.* 2004). The call is very quiet and can only be easily heard when in close proximity or when a large number of males is calling.

Distribution

It has a very restricted distribution in the southern end of the Grootwinterhoek Mountains north of Tulbagh.

Habitat

Well-developed seeps with dense restios.

Breeding

Two clutches have been recorded with 5 and 7 eggs respectively.

***Arthroleptella landdrosia* Dawood & Channing 2000**

Landdros moss frog

Photographs

Figure 91. *Arthroleptella landdrosia* male from Landdroskop in the Hottentots-Holland Mountains.

Figure 92. *Arthroleptella landdrosia* male from Swartboskloof in the Hottentots-Holland Mountains.

Figure 93. *Arthroleptella landdrosia* male from Jonkershoek, north of the Eerste River.

Figure 94. *Arthroleptella landdrosia* male from Simonsberg Mountain.

Figure 95. *Arthroleptella landdrosia* male from Groenlandberg Mountain.

Figure 96. *Arthroleptella landdrosia* male from Houwhoek Mountain.

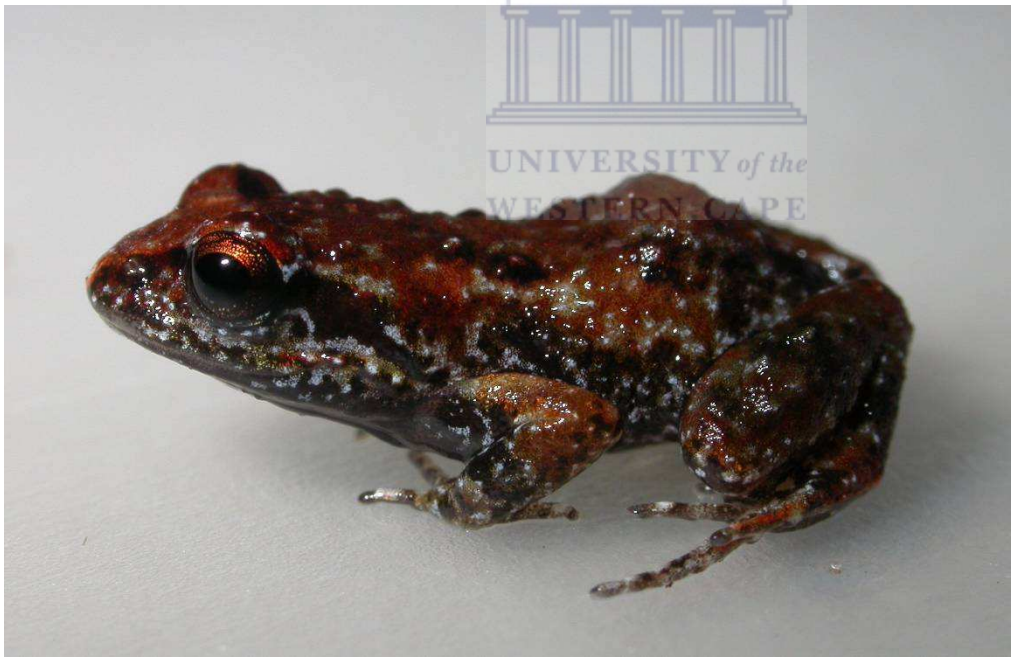

Figure 97. *Arthroleptella landdrosia* male from Houwhoek Mountain.

Appearance

Colouration is very varied but normally consists of dark brown to black blotches and spots on a background colour that varies from dark brown, through red-brown, yellow-brown to grey. A black stripe extends from the nares to the angle of the jaw or to the insertion of the arm. The snout is

rounded. The toe tips are expanded and finger tips may be slightly expanded. The outer metatarsal tubercle and inner metatarsal are poorly developed. The dorsal skin has variably developed lumps and ridges. Males have the throat and chest region black; females are white below.

Call

The call is a long, harsh series of clicks.

Distribution

It is known from the Simonsberg, Jonkershoek, Hottentots-Holland, Helderberg, Groenland and Houwhoek Mountains.

Habitat

Very well-developed mountain seeps, seepages on cliff faces and in wet areas under scree.

Breeding

Calling occurs over a very extended period in suitable weather and has been recorded in all months except February and March. Clutch size is unknown.

***Arthroleptella drewesii* Channing, Hendricks & Dawood 1994**

Drewes' moss frog

Photographs

Figure 98. *Arthroleptella drewesii* male from Kleinrivier Mountains.

Figure 99. *Arthroleptella drewesii* male from Babilonstoring Mountain.

Appearance

Colouration is varied but often consists of a irregular pattern of fine dark brown blotches on a paler background dorsally. Some individuals may be almost uniformly black brown. A fine, pale vertebral strip may be present. Males are dark grey to black on the head and chest. Females are immaculate white below. The finger and toe tips are expanded.

Call

A long, melodious set of clicks.

Distribution

This species is only known from the Kleinrivier and Babilonstoring Mountains.

Habitat

Various seepage areas on mountain slopes.

Breeding

Calling has been recorded in the wet winter months from June to September. Clutch size is unknown.

***Arthroleptella sp.* A West and East**

Photographs

Figure 100. *Arthroleptella sp*. A West male from the farm Amanzi in the Riviersonderend Mountains.

Figure 101. *Arthroleptella sp*. A West male from the farm Amanzi in the Riviersonderend Mountains.

Figure 102. *Arthroleptella sp*. A East male from Die Galg in the Riviersonderend Mountains.

Figure 103. *Arthroleptella sp*. A East male from Kanonberg in the Riviersonderend Mountains.

Figure 104. *Arthroleptella sp*. A East male from Kanonberg in the Riviersonderend Mountains.

Figure 105. *Arthroleptella sp.* A East male from the farm Twistniet in the Riviersonderend Mountains.

Appearance

Finger and toe tips expanded. Colouration is in various shades of brown with irregular darker markings dorsally. The throats and chests of breeding males are black, this colour fades to grey mottling posteriorly. The vocal sac is well-developed. Females are white below.

Call

A series of clicks.

Distribution

Occurs on the southern slopes of the Riviersonderend Mountains.

Habitat

Seepages and wet cliffs.

Breeding

Calling is known to from May to November. Clutch size is unknown.

***Arthroleptella sp.* B**

Photographs

Figure 106. *Arthroleptella sp.* B male from Zachariashoek, Limietberg Nature Reserve.

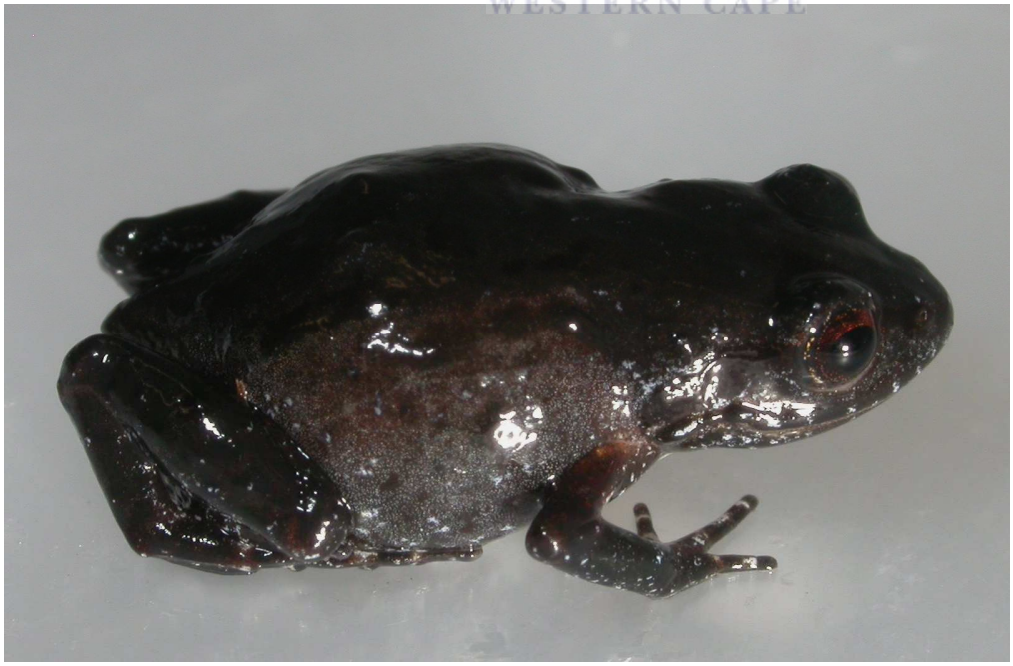

Figure 107. *Arthroleptella sp. B* female from Zachariashoek, Limietberg Nature Reserve. Note clearly visible tympanum.

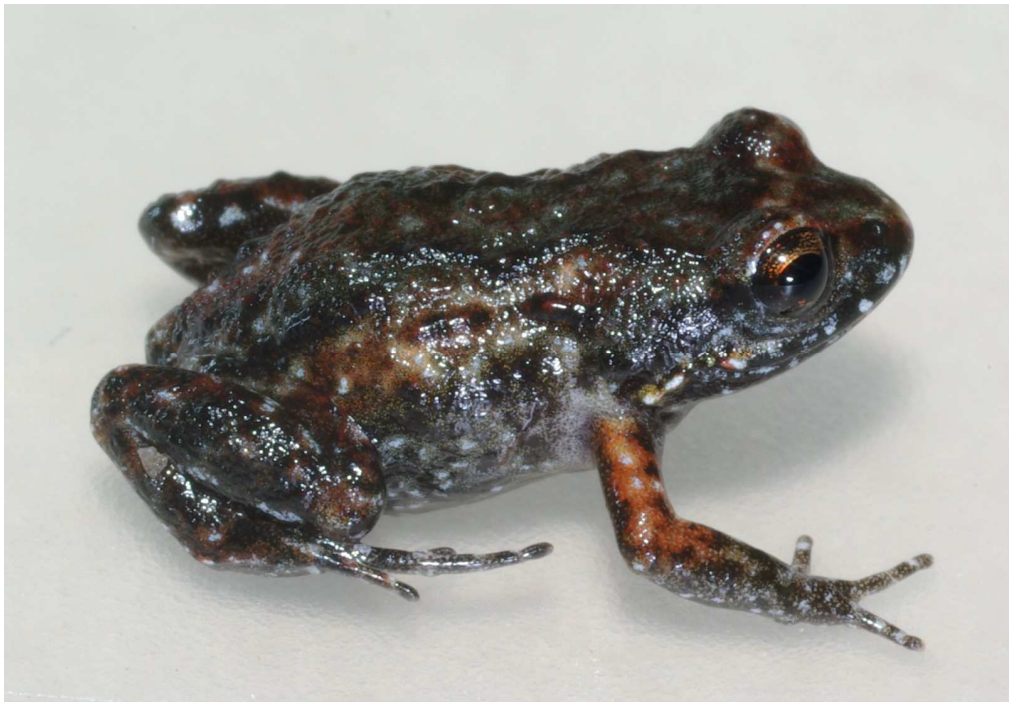

Figure 108. *Arthroleptella sp. B* male from Du Toitskloof Mountains.

Appearance

This species is morphologically very similar to *A. bicolor* in that is also has a squat appearance with a broadly rounded snout. Finger and toe tips are not expanded or slightly expanded. The single female captured displays a clearly visible tympanum, a character not known from males or females of any other species of *Arthroleptella* (Figure 107) although the diagnostic value of this character is unknown. Colour varies above from orange-brown to black. There may be irregular darker marks dorsally including bands on the upper surfaces of the legs. A dark mark may extend from the eye to the arm. The lips are is dark grey with white flecks.

Call

A set of clicks.

Distribution

It is known from the Klein Drakenstein, Du Toitskloof Mountains and Villiersdorp Mountains.

Habitat

Well-developed mountain seeps with dense restios vegetation.

Breeding

Calling has been recorded from May to November. The only known clutch size consisted of 8 eggs.

***Arthroleptella sp.* C**

Photographs

Figure 109. *Arthroleptella sp.* C male from the Kogelberg Nature Reserve.

Figure 110. *Arthroleptella sp.* C male from the Koëlberg Mountains.

Figure 111. *Arthroleptella sp.* C male from the Koëlberg Mountains.

Figure 112. *Arthroleptella sp.* C male from Betty's Bay.

Call

A series of clicks.

Appearance

Snout rounded. Finger and toe tips expanded. Variably marked in black to dark brown on a paler brown to red-brown background. Males are black on the throat and chest to entirely black below. Females unknown.

Distribution

On the Koëlberg and Kogelberg Mountains, including an isolated population at sea level at Betty's Bay.

Habitat

Well developed mountain seeps. A single population is known from a seep in a coastal dune slack.

Breeding

Calling is known from July to October but is likely to extend beyond these months. Clutch size is unknown.

Appendix 2. Names and geographic coordinates of localities sampled. See Appendix 4 for a map.

Field Specimen Number	Taxon	Locality	Lat	Long
AAT-210	*Arthroleptella bicolor*	Bainskloof	-33.61	19.11
AAT-260	*Arthroleptella bicolor*	Bainskloof	-33.60	19.11
AAT-369	*Arthroleptella bicolor*	Bainskloof	-33.61	19.11
AAT-386	*Arthroleptella bicolor*	Bainskloof	-33.61	19.11
AAT-246	*Arthroleptella bicolor*	Bastiaanskloof, Limietberg	-33.52	19.14
AAT-349	*Arthroleptella bicolor*	Observation Peak	-33.62	19.13
AAT-251	*Arthroleptella bicolor*	Waterval Nature Reserve, Suurvlakte plantation.	-33.39	19.10
AAT-252	*Arthroleptella bicolor*	Waterval Nature Reserve, Suurvlakte plantation.	-33.39	19.10
AAT-292	*Arthroleptella drewesii*	Babilonstoring N.R., eastern side.	-34.33	19.24
AAT-294	*Arthroleptella drewesii*	Babilonstoring N.R., eastern side.	-34.33	19.24
AAT-211	*Arthroleptella drewesii*	Fernkloof	-34.39	19.27
AAT-212	*Arthroleptella drewesii*	Fernkloof	-34.39	19.27
AAT-364	*Arthroleptella drewesii*	Fernkloof	-34.38	19.28
AAT-365	*Arthroleptella drewesii*	Fernkloof	-34.39	19.30
AAT-366	*Arthroleptella drewesii*	Fernkloof	-34.38	19.29
AAT-367	*Arthroleptella drewesii*	Fernkloof	-34.38	19.28
AAT-368	*Arthroleptella drewesii*	Fernkloof	-34.38	19.28
AAT-226	*Arthroleptella drewesii*	Vogelgat N.R.	-34.40	19.32
AAT-227	*Arthroleptella drewesii*	Vogelgat N.R.	-34.40	19.32
AAT-228	*Arthroleptella drewesii*	Vogelgat N.R.	-34.40	19.32
AAT-309	*Arthroleptella landdrosia*	Delheim, Simonsberg	-33.87	18.90
AAT-270	*Arthroleptella landdrosia*	Groenlandberg	-34.11	19.08
AAT-271	*Arthroleptella landdrosia*	Groenlandberg	-34.12	19.10
AAT-272	*Arthroleptella landdrosia*	Groenlandberg	-34.13	19.12

Field Specimen Number	Taxon	Locality	Lat	Long
AAT-274	*Arthroleptella landdrosia*	Groenlandberg	-34.13	19.11
AAT-381	*Arthroleptella landdrosia*	Groenlandberg	-34.11	19.08
AAT-382	*Arthroleptella landdrosia*	Groenlandberg	-34.13	19.11
AAT-383	*Arthroleptella landdrosia*	Groenlandberg	-34.13	19.11
AAT-224	*Arthroleptella landdrosia*	Helderberg	-34.02	18.89
AAT-225	*Arthroleptella landdrosia*	Helderberg	-34.02	18.89
AAT-261	*Arthroleptella landdrosia*	Helderberg	-34.02	18.89
AAT-262	*Arthroleptella landdrosia*	Houwhoek Mountain	-34.20	19.17
AAT-263	*Arthroleptella landdrosia*	Houwhoek Mountain	-34.19	19.17
AAT-264	*Arthroleptella landdrosia*	Houwhoek Mountain	-34.19	19.17
AAT-265	*Arthroleptella landdrosia*	Houwhoek Mountain	-34.19	19.17
AAT-266	*Arthroleptella landdrosia*	Houwhoek Mountain	-34.19	19.17
AAT-267	*Arthroleptella landdrosia*	Houwhoek Mountain	-34.19	19.17
AAT-353	*Arthroleptella landdrosia*	Houwhoek Mountain	-34.19	19.17
AAT-354	*Arthroleptella landdrosia*	Houwhoek Mountain	-34.20	19.17
AAT-355	*Arthroleptella landdrosia*	Houwhoek Mountain	-34.20	19.17
AAT-357	*Arthroleptella landdrosia*	Houwhoek Mountain	-34.20	19.17
AAT-385	*Arthroleptella landdrosia*	Houwhoek Mountain	-34.20	19.17
AAT-316	*Arthroleptella landdrosia*	Jonkershoek Plantation	-33.98	18.94
AAT-317	*Arthroleptella landdrosia*	Jonkershoek Plantation	-33.98	18.93
AAT-326	*Arthroleptella landdrosia*	Jonkershoek Plantation	-33.96	18.93
AAT-372	*Arthroleptella landdrosia*	Jonkershoek Plantation	-33.98	18.97
AAT-373	*Arthroleptella landdrosia*	Jonkershoek Plantation	-33.98	18.96
AAT-208	*Arthroleptella landdrosia*	Landdroskop	-34.05	19.01
AAT-273	*Arthroleptella landdrosia*	Landdroskop	-34.05	19.00
AAT-352	*Arthroleptella landdrosia*	Landdroskop	-34.05	19.01

Field Specimen Number	Taxon	Locality	Lat	Long
AAT-238	*Arthroleptella landdrosia*	SE Slopes of Simonsberg.	-33.90	18.93
AAT-239	*Arthroleptella landdrosia*	SE Slopes of Simonsberg.	-33.90	18.95
AAT-244	*Arthroleptella landdrosia*	Simonsberg lower slopes, Delheim Wine Estate.	-33.87	18.90
AAT-258	*Arthroleptella landdrosia*	Simonsberg lower slopes, Delheim Wine Estate.	-33.87	18.90
AAT-259	*Arthroleptella landdrosia*	Simonsberg lower slopes, Delheim Wine Estate.	-33.87	18.90
AAT-209	*Arthroleptella landdrosia*	Swartboskloof	-34.00	18.96
AAT-362	*Arthroleptella landdrosia*	Swartboskloof	-33.99	18.94
AAT-332	*Arthroleptella lightfooti*	Cape Point	-34.27	18.46
AAT-333	*Arthroleptella lightfooti*	Cape Point	-34.27	18.46
AAT-334	*Arthroleptella lightfooti*	Cape Point	-34.27	18.46
AAT-335	*Arthroleptella lightfooti*	Cape Point	-34.27	18.44
AAT-336	*Arthroleptella lightfooti*	Cape Point, Olifantsbos	-34.26	18.39
AAT-253	*Arthroleptella lightfooti*	Cecilia Plantation, Constantia	-34.01	18.42
AAT-254	*Arthroleptella lightfooti*	Cecilia Plantation, Constantia	-34.01	18.42
AAT-255	*Arthroleptella lightfooti*	Cecilia Plantation, Constantia	-34.01	18.41
AAT-256	*Arthroleptella lightfooti*	Cecilia Plantation, Constantia	-34.01	18.41
AAT-313	*Arthroleptella lightfooti*	Kirstenbosch	-33.99	18.43
AAT-269	*Arthroleptella lightfooti*	Plateau Road, Cape Town	-34.20	18.40
AAT-337	*Arthroleptella lightfooti*	Silvermine	-34.08	18.40
AAT-338	*Arthroleptella lightfooti*	Silvermine	-34.08	18.40
AAT-249	*Arthroleptella lightfooti*	Silvermine area.	-34.13	18.43
AAT-250	*Arthroleptella lightfooti*	Silvermine area.	-34.10	18.44
AAT-344	*Arthroleptella lightfooti*	Table Mountain, above Nursery Ravine	-33.98	18.41
AAT-345	*Arthroleptella lightfooti*	Table Mountain, above Nursery Ravine	-33.98	18.41
AAT-339	*Arthroleptella lightfooti*	Table Mountain, back table road	-34.00	18.42
AAT-340	*Arthroleptella lightfooti*	Table Mountain, back table road	-34.00	18.41

Field Specimen Number	Taxon	Locality	Lat	Long
AAT-341	*Arthroleptella lightfooti*	Table Mountain, back table road	-34.00	18.41
AAT-342	*Arthroleptella lightfooti*	Table Mountain, back table road	-34.00	18.41
AAT-346	*Arthroleptella lightfooti*	Table Mountain, Nursery Ravine	-33.99	18.42
AAT-343	*Arthroleptella lightfooti*	Table Mountain, Skeleton Gorge	-33.98	18.42
AAT-257	*Arthroleptella lightfooti*	Tokai Plantation	-34.06	18.41
AAT-201	*Arthroleptella rugosa*	Caledon	-34.21	19.54
AAT-202	*Arthroleptella rugosa*	Caledon	-34.21	19.54
AAT-213	*Arthroleptella rugosa*	Caledon	-34.21	19.54
AAT-214	*Arthroleptella rugosa*	Caledon	-34.21	19.54
AAT-215	*Arthroleptella rugosa*	Caledon	-34.21	19.53
AAT-216	*Arthroleptella rugosa*	Caledon	-34.21	19.53
AAT-217	*Arthroleptella rugosa*	Caledon	-34.21	19.53
AAT-218	*Arthroleptella rugosa*	Caledon	-34.21	19.53
AAT-308	*Arthroleptella rugosa*	Caledon	-34.21	19.53
AAT-319	*Arthroleptella rugosa*	Caledon	-34.20	19.48
AAT-320	*Arthroleptella rugosa*	Caledon	-34.20	19.48
AAT-321	*Arthroleptella rugosa*	Caledon	-34.20	19.48
AAT-205	*Arthroleptella sp. A East*	Die Galg	-34.01	19.71
AAT-315	*Arthroleptella sp. A East*	Die Galg	-34.01	19.71
AAT-374	*Arthroleptella sp. A East*	Kanonberg, Greyton	-34.03	19.61
AAT-375	*Arthroleptella sp. A East*	Kanonberg, Greyton	-34.02	19.61
AAT-376	*Arthroleptella sp. A East*	Kanonberg, Greyton	-34.02	19.61
AAT-377	*Arthroleptella sp. A East*	Kanonberg, Greyton	-34.02	19.60
AAT-379	*Arthroleptella sp. A East*	Kanonberg, Greyton	-34.03	19.61
AAT-358	*Arthroleptella sp. A East*	Twistniet, Riviersonderend Mountains	-34.11	19.87
AAT-359	*Arthroleptella sp. A East*	Twistniet, Riviersonderend Mountains	-34.11	19.87

Field Specimen Number	Taxon	Locality	Lat	Long
AAT-360	*Arthroleptella sp. A East*	Twistniet, Riviersonderend Mountains	-34.11	19.87
AAT-278	*Arthroleptella sp. A West*	Amanzi, Riviersonderend	-34.04	19.46
AAT-279	*Arthroleptella sp. A West*	Amanzi, Riviersonderend	-34.04	19.46
AAT-280	*Arthroleptella sp. A West*	Amanzi, Riviersonderend	-34.04	19.45
AAT-300	*Arthroleptella sp. A West*	Amanzi, Riviersonderend	-34.04	19.46
AAT-301	*Arthroleptella sp. A West*	Amanzi, Riviersonderend	-34.04	19.46
AAT-302	*Arthroleptella sp. A West*	Amanzi, Riviersonderend	-34.04	19.46
AAT-275	*Arthroleptella sp. A West*	Jonaskop	-33.98	19.49
AAT-276	*Arthroleptella sp. A West*	Jonaskop	-33.98	19.49
AAT-299	*Arthroleptella sp. B*	Aasvogelberg, behind Villiersdorp	-33.93	19.24
AAT-303	*Arthroleptella sp. B*	Aasvogelberg, behind Villiersdorp	-33.93	19.24
AAT-304	*Arthroleptella sp. B*	Aasvogelberg, behind Villiersdorp	-33.93	19.24
AAT-200	*Arthroleptella sp. B*	Du Toitskloof	-33.69	19.10
AAT-387	*Arthroleptella sp. B*	Du Toitskloof	-33.69	19.10
AAT-277	*Arthroleptella sp. B*	Fizantakraal, Elandspad Rd, Du Toitskloof	-33.78	19.16
AAT-307	*Arthroleptella sp. B*	Fizantakraal, Elandspad Rd, Du Toitskloof	-33.78	19.16
AAT-350	*Arthroleptella sp. B*	Hawequa Mountains	-33.69	19.10
AAT-322	*Arthroleptella sp. B*	Zachariashoek, Kleindrakenstein Mountains	-33.81	19.03
AAT-323	*Arthroleptella sp. B*	Zachariashoek, Kleindrakenstein Mountains	-33.80	19.03
AAT-324	*Arthroleptella sp. B*	Zachariashoek, Kleindrakenstein Mountains	-33.80	19.03
AAT-325	*Arthroleptella sp. B*	Zachariashoek, Kleindrakenstein Mountains	-33.80	19.03
AAT-229	*Arthroleptella sp. C*	Betty's Bay	-34.36	18.91
AAT-230	*Arthroleptella sp. C*	Betty's Bay	-34.36	18.91
AAT-231	*Arthroleptella sp. C*	Betty's Bay	-34.36	18.91
AAT-232	*Arthroleptella sp. C*	Betty's Bay	-34.36	18.91
AAT-233	*Arthroleptella sp. C*	Betty's Bay	-34.36	18.91

Field Specimen Number	Taxon	Locality	Lat	Long
AAT-234	*Arthroleptella sp. C*	Betty's Bay	-34.36	18.91
AAT-288	*Arthroleptella sp. C*	Koëlberg above Steenbras dam	-34.20	18.92
AAT-289	*Arthroleptella sp. C*	Koëlberg above Steenbras dam	-34.21	18.93
AAT-296	*Arthroleptella sp. C*	Koëlberg above Steenbras dam	-34.21	18.93
AAT-305	*Arthroleptella sp. C*	Koëlberg above Steenbras dam	-34.21	18.93
AAT-242	*Arthroleptella sp. C*	Kogelberg, Oudebosch	-34.34	18.94
CNCH-6746	*Arthroleptella subvoce*	Groot Winterhoek Wilderness Area.	-33.00	19.06
CNCH-6747	*Arthroleptella subvoce*	Groot Winterhoek Wilderness Area.	-33.00	19.06
CNCH-6748	*Arthroleptella subvoce*	Groot Winterhoek Wilderness Area.	-33.00	19.06
CNCH-6749	*Arthroleptella subvoce*	Groot Winterhoek Wilderness Area.	-33.02	19.07
AAT-281	*Arthroleptella subvoce*	Grootwinterhoek Wilderness Area	-33.02	19.07
AAT-282	*Arthroleptella subvoce*	Grootwinterhoek Wilderness Area	-33.00	19.06
AAT-306	*Arthroleptella subvoce*	Grootwinterhoek Wilderness Area	-33.02	19.07
AAT-247	*Arthroleptella subvoce*	Sneeugat Trail	-33.15	19.17
AAT-248	*Arthroleptella subvoce*	Sneeugat Trail	-33.15	19.17
AAT-347	*Arthroleptella villiersi*	Aasfontein, Soetanysberg	-34.77	19.87
AAT-348	*Arthroleptella villiersi*	Aasfontein, Soetanysberg	-34.76	19.89
AAT-206	*Arthroleptella villiersi*	Babilonstoring	-34.32	19.22
AAT-207	*Arthroleptella villiersi*	Babilonstoring	-34.33	19.21
AAT-290	*Arthroleptella villiersi*	Babilonstoring N.R., eastern side.	-34.32	19.24
AAT-291	*Arthroleptella villiersi*	Babilonstoring N.R., eastern side.	-34.33	19.24
AAT-293	*Arthroleptella villiersi*	Babilonstoring N.R., eastern side.	-34.33	19.24
AAT-235	*Arthroleptella villiersi*	Betty's Bay	-34.36	18.91
AAT-236	*Arthroleptella villiersi*	Betty's Bay	-34.36	18.91
AAT-237	*Arthroleptella villiersi*	Betty's Bay	-34.36	18.91
AAT-361	*Arthroleptella villiersi*	Bredasdorpberg	-34.52	19.88

Field Specimen Number	Taxon	Locality	Lat	Long
AAT-245	*Arthroleptella villiersi*	Franschhoek Pass, NE side	-33.93	19.16
AAT-378	*Arthroleptella villiersi*	Greyton nature Reserve	-34.04	19.61
AAT-380	*Arthroleptella villiersi*	Groenlandberg	-34.11	19.08
AAT-327	*Arthroleptella villiersi*	GrootDrakenstein Mountains	-33.91	19.04
AAT-328	*Arthroleptella villiersi*	Groot Drakenstein Mountains	-33.92	19.04
AAT-329	*Arthroleptella villiersi*	Groot Drakenstein Mountains	-33.92	19.03
AAT-330	*Arthroleptella villiersi*	Groot Drakenstein Mountains	-33.92	19.03
AAT-331	*Arthroleptella villiersi*	Groot Drakenstein Mountains	-33.92	19.03
AAT-268	*Arthroleptella villiersi*	Houwhoek Mountain	-34.20	19.17
AAT-356	*Arthroleptella villiersi*	Houwhoek Mountain	-34.20	19.17
AAT-318	*Arthroleptella villiersi*	Jonkershoek Plantation	-33.99	18.94
AAT-298	*Arthroleptella villiersi*	Junction of R44 to Kleinmond and R43 to Hermanus.	-34.30	19.14
AAT-297	*Arthroleptella villiersi*	Kleinriviersberg, eastern end.	-34.39	19.60
AAT-283	*Arthroleptella villiersi*	Koëlberg above Steenbras dam	-34.16	18.93
AAT-284	*Arthroleptella villiersi*	Koëlberg above Steenbras dam	-34.21	18.85
AAT-285	*Arthroleptella villiersi*	Koëlberg above Steenbras dam	-34.20	18.86
AAT-286	*Arthroleptella villiersi*	Koëlberg above Steenbras dam	-34.20	18.91
AAT-287	*Arthroleptella villiersi*	Koëlberg above Steenbras dam	-34.22	18.88
AAT-295	*Arthroleptella villiersi*	Koëlberg above Steenbras dam	-34.21	18.93
AAT-240	*Arthroleptella villiersi*	Kogelberg, Oudebosch	-34.32	18.96
AAT-243	*Arthroleptella villiersi*	Kogelberg, Oudebosch	-34.33	18.93
AAT-241	*Arthroleptella villiersi*	Kogelberg, Palmiet Valley	-34.29	18.93
AAT-312	*Arthroleptella villiersi*	Landdroskop	-34.05	19.01
AAT-351	*Arthroleptella villiersi*	Landdroskop	-34.05	19.01
AAT-219	*Arthroleptella villiersi*	Paarl Rock	-33.75	18.94
AAT-220	*Arthroleptella villiersi*	Paarl Rock	-33.73	18.93

Field Specimen Number	Taxon	Locality	Lat	Long
AAT-221	*Arthroleptella villiersi*	Paarl Rock	-33.73	18.93
AAT-222	*Arthroleptella villiersi*	Paarl Rock	-33.73	18.93
AAT-223	*Arthroleptella villiersi*	Paarl Rock	-33.73	18.93
AAT-310	*Arthroleptella villiersi*	Paarl Rock	-33.73	18.93
AAT-311	*Arthroleptella villiersi*	Paarl Rock	-33.73	18.93
AAT-384	*Arthroleptella villiersi*	Rusbos, Hottentots-Holland Nature Reserve	-33.97	19.16
AAT-314	*Arthroleptella villiersi*	Steenboksberg	-34.32	19.44
AAT-363	*Arthroleptella villiersi*	Swartboskloof	-33.99	18.94

Appendix 3. Detailed AMOVA results.

Results for all Arthroleptella:

```
================================================================================
 Comparisons of pairs of population samples
================================================================================
List of labels for population samples used below:
------------------------------------------------

Label     Population name
-----     ---------------
  1:      sp. C
  2:      landdrosia
  3:      drewesii
  4:      sp. A
  5:      sp. B
  6:      bicolor
  7:      subvoce
  8:      rugosa
  9:      villiersi
  10:     lightfooti
```

```
------------------------
Population pairwise FSTs
------------------------

Distance method: Pairwise difference
                    1         2         3         4         5         6         7         8         9        10
           1   0.00000
           2   0.65116   0.00000
           3   0.94379   0.63690   0.00000
           4   0.58046   0.55511   0.48348   0.00000
           5   0.87856   0.74667   0.88595   0.51897   0.00000
           6   0.90553   0.75955   0.90458   0.55059   0.79821   0.00000
           7   0.89801   0.76964   0.91138   0.53106   0.80814   0.65442   0.00000
           8   0.97866   0.92677   0.98159   0.86626   0.95946   0.96237   0.96362   0.00000
           9   0.97368   0.94175   0.97700   0.90003   0.96312   0.96486   0.96182   0.92464   0.00000
          10   0.96677   0.92284   0.96805   0.86253   0.95046   0.95194   0.94965   0.90856   0.78047   0.00000

------------
FST P values
------------

Number of permutations : 110
```

1	2	3	4	5	6	7	8	9	10
1	*								
2	0.00000+- 0.0000	*							
3	0.00000+- 0.0000	0.00000+- 0.0000	*						
4	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	*					
5	0.00000+- 0.0000	0.00000+- 0.0000	0.00901+- 0.0091	0.00000+- 0.0000	*				
6	0.00000+- 0.0000	0.00000+- 0.0000	0.02703+- 0.0139	0.00000+- 0.0000	0.00901+- 0.0091	*			

1	2	3	4	5	6	7	8	9	10	
7	0.00000+- 0.0000	0.00000+- 0.0000	0.02703+- 0.0139	0.00000+- 0.0000	0.00000+- 0.0000	0.00901+- 0.0091	*			
8	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	0.02703+- 0.0139	0.00000+- 0.0000	*		
9	0.00000+- 0.0000	0.00000+- 0.0000	0.00901+- 0.0091	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	*	
10	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	0.00000+- 0.0000	*

Matrix of significant Fst P values

Significance Level=0.0500

Number of permutations : 110

	1	2	3	4	5	6	7	8	9	10
1		+	+	+	+	+	+	+	+	+
2	+		+	+	+	+	+	+	+	+
3	+	+		+	+	+	+	+	+	+
4	+	+	+		+	+	+	+	+	+
5	+	+	+	+		+	+	+	+	+
6	+	+	+	+	+		+	+	+	+
7	+	+	+	+	+	+		+	+	+
8	+	+	+	+	+	+	+		+	+
9	+	+	+	+	+	+	+	+		+
10	+	+	+	+	+	+	+	+	+	

Matrix of Slatkin linearized FSTs as t/M=FST/(1-FST)

(M=N for haploid data, M=2N for diploid data)

	1	2	3	4	5	6	7	8	9	10
1	0.00000									
2	1.86660	0.00000								
3	16.79048	1.75406	0.00000							
4	1.38354	1.24775	0.93602	0.00000						
5	7.23449	2.94735	7.76833	1.07887	0.00000					
6	9.58592	3.15895	9.47949	1.22515	3.95566	0.00000				
7	8.80487	3.34111	10.28431	1.13246	4.21220	1.89366	0.00000			
8	45.85013	12.65568	53.32083	6.47700	23.66928	25.57143	26.48622	0.00000		
9	37.00106	16.16883	42.47917	9.00316	26.11139	27.45833	25.19375	12.26893	0.00000	
10	29.09104	11.96032	30.29825	6.27419	19.18374	19.80829	18.86111	9.93590	3.55529	0.00000

Matrix of M values (M=Nm for haploid data, M=2Nm for diploid data)

	1	2	3	4	5	6	7	8	9	10
1										
2	0.26787									
3	0.02978	0.28505								
4	0.36139	0.40072	0.53418							
5	0.06911	0.16964	0.06436	0.46345						
6	0.05216	0.15828	0.05275	0.40811	0.12640					
7	0.05679	0.14965	0.04862	0.44152	0.11870	0.26404				
8	0.01091	0.03951	0.00938	0.07720	0.02112	0.01955	0.01888			
9	0.01351	0.03092	0.01177	0.05554	0.01915	0.01821	0.01985	0.04075		
10	0.01719	0.04180	0.01650	0.07969	0.02606	0.02524	0.02651	0.05032	0.14064	

==========

== Exact Test of Sample Differentiation Based on Haplotype Frequencies

==========

List of labels for population samples used below:

Label	Population name
1	sp. C
2	landdrosia
3	drewesii
4	sp. A
5	sp. B
6	bicolor
7	subvoce
8	villiersi
9	lightfooti
10	rugosa

Global test of differentiation among sample:

Non-differentiation: Exact P value = 0.00000 +- 0.00000 (6000 Markov steps done)

Differentiation test between all pairs of samples:

Markov chain length : 10000 steps)

Non-differentiation exact P values :

	1	2	3	4	5	6	7	8	9
2	0.00175+- 0.0018								
3	0.01510+- 0.0024	0.15050+- 0.0167							
4	0.00505+- 0.0019	0.00085+- 0.0005	0.11265+- 0.0074						
5	0.00090+- 0.0006	0.00105+- 0.0009	0.04585+- 0.0055	0.00185+- 0.0009					
6	0.01675+- 0.0030	0.10285+- 0.0181	0.46530+- 0.0155	0.09670+- 0.0117	0.05505+- 0.0085				
7	0.00315+- 0.0010	0.02265+- 0.0081	0.11875+- 0.0100	0.01430+- 0.0032	0.00960+- 0.0022	0.20630+- 0.0140			
8	0.00000+- 0.0000	0.00000+- 0.0000	0.02140+- 0.0031	0.00000+- 0.0000	0.00150+- 0.0016	0.01350+- 0.0050	0.00055+- 0.0005		
9	0.00465+- 0.0021	0.02135+- 0.0066	0.12955+- 0.0087	0.01965+- 0.0044	0.00950+- 0.0022	0.20620+- 0.0129	0.04840+- 0.0032	0.00075+- 0.0005	
10	0.00200+- 0.0011	0.03145+- 0.0070	0.08380+- 0.0068	0.03935+- 0.0089	0.01415+- 0.0018	0.19325+- 0.0115	0.04285+- 0.0040	0.00065+- 0.0007	0.03870+- 0.0045

Table of significant differences (significance level=0.0500):

	1	2	3	4	5	6	7	8	9	10
1		+	+	+	+	+	+	+	+	+
2	+		-	+	+	-	+	+	+	+
3	+	-		-	+	-	-	+	-	-
4	+	+	-		+	-	+	+	+	+
5	+	+	+	+		-	+	+	+	+
6	+	-	-	-	-		-	+	-	-
7	+	+	-	+	+	-		+	+	+
8	+	+	+	+	+	+	+		+	+
9	+	+	-	+	+	-	+	+		+
10	+	+	-	+	+	-	+	+	+	

Histogram of the number of significant different populations (significance level=0.0500):

1	2	3	4	5	6	7	8	9	10
9	7	3	7	8	2	7	9	7	7

Results for Chirping clade only

```
================================================================================
== Comparisons of pairs of population samples
================================================================================

List of labels for population samples used below:
------------------------------------------------

Label     Population name
-----     ---------------
  1:      villiersi
  2:      lightfooti
  3:      rugosa

------------------------
Population pairwise FSTs
------------------------

Distance method: Pairwise difference
                     1         2         3
           1   0.00000
           2   0.78047   0.00000
           3   0.92464   0.90856   0.00000
```

```
------------
FST P values
------------

Number of permutations : 110

                     1                2                3
          1          *
          2   0.00000+-0.0000          *
          3   0.00000+-0.0000   0.00000+-0.0000          *

------------
Matrix of significant Fst P values
Significance Level=0.0500
------------

Number of permutations : 110

                    1         2         3
          1                   +         +
          2         +                   +
          3         +         +

--------------------------------------------------
Matrix of Slatkin linearized FSTs as t/M=FST/(1-FST)
(M=N for haploid data, M=2N for diploid data)
--------------------------------------------------

                    1         2         3
          1   0.00000
          2   3.55529   0.00000
          3  12.26893   9.93590   0.00000
```

```
---------------------------------------------------------------
Matrix of M values (M=Nm for haploid data, M=2Nm for diploid data)
---------------------------------------------------------------

                    1         2         3
          1
          2   0.14064
          3   0.04075   0.05032



================================================================================
== Exact Test of Sample Differentiation Based on Haplotype Frequencies
================================================================================

List of labels for population samples used below:
------------------------------------------------

Label     Population name
-----     ---------------
  1       villiersi
  2       lightfooti
  3       rugosa

--------------------------------------------------------------------------
Global test of differentiation among sample:
----------------------------------------

 Non-differentiation: Exact P value = 0.00000 +- 0.00000  (6000 Markov steps done)

--------------------------------------------------------------------------
Differentiation test between all pairs of samples:
-------------------------------------------------

Markov chain length : 10000 steps)
```

Non-differentiation exact P values :

	1	2
2	0.00395+-0.0017	
3	0.00105+-0.0007	0.03945+-0.0040

Table of significant differences (significance level=0.0500):

	1	2	3
1		+	+
2	+		+
3	+	+	

Histogram of the number of significant different populations (significance level=0.0500):

1	2	3
2	2	2

Results for Clicking clade only

```
================================================================================
== Comparisons of pairs of population samples
================================================================================

List of labels for population samples used below:
------------------------------------------------

Label     Population name
-----     ---------------
  1:      sp. C
  2:      landdrosia
  3:      drewesii
  4:      sp. A
  5:      sp. B
  6:      bicolor
  7:      subvoce

------------------------
Population pairwise FSTs
------------------------

Distance method: Pairwise difference
                     1         2         3         4         5         6         7
           1   0.00000
           2   0.65116   0.00000
           3   0.94379   0.63690   0.00000
           4   0.58046   0.55511   0.48348   0.00000
           5   0.87856   0.74667   0.88595   0.51897   0.00000
           6   0.90553   0.75955   0.90458   0.55059   0.79821   0.00000
           7   0.89801   0.76964   0.91138   0.53106   0.80814   0.65442   0.00000
```

```
------------
FST P values
------------

Number of permutations : 110
```

	1	2	3	4	5	6	7
1	*						
2	0.00000+-0.0000	*					
3	0.01802+-0.0121	0.00000+-0.0000	*				
4	0.00000+-0.0000	0.00000+-0.0000	0.00000+-0.0000	*			
5	0.00000+-0.0000	0.00000+-0.0000	0.00901+-0.0091	0.00000+-0.0000	*		
6	0.00000+-0.0000	0.00000+-0.0000	0.01802+-0.0121	0.00000+-0.0000	0.00000+-0.0000	*	
7	0.00000+-0.0000	0.00000+-0.0000	0.01802+-0.0121	0.00000+-0.0000	0.00000+-0.0000	0.00000+-0.0000	*


```
------------
Matrix of significant Fst P values
Significance Level=0.0500
------------

Number of permutations : 110
```

	1	2	3	4	5	6	7
1		+	+	+	+	+	+
2	+		+	+	+	+	+
3	+	+		+	+	+	+
4	+	+	+		+	+	+
5	+	+	+	+		+	+
6	+	+	+	+	+		+
7	+	+	+	+	+	+	

```
------------------------------------------------
Matrix of Slatkin linearized FSTs as t/M=FST/(1-FST)
(M=N for haploid data, M=2N for diploid data)
------------------------------------------------
```

	1	2	3	4	5	6	7
1	0.00000						
2	1.86660	0.00000					
3	16.79048	1.75406	0.00000				
4	1.38354	1.24775	0.93602	0.00000			
5	7.23449	2.94735	7.76833	1.07887	0.00000		
6	9.58592	3.15895	9.47949	1.22515	3.95566	0.00000	
7	8.80487	3.34111	10.28431	1.13246	4.21220	1.89366	0.00000

```
---------------------------------------------------------------
Matrix of M values (M=Nm for haploid data, M=2Nm for diploid data)
---------------------------------------------------------------
```

	1	2	3	4	5	6	7
1							
2	0.26787						
3	0.02978	0.28505					
4	0.36139	0.40072	0.53418				
5	0.06911	0.16964	0.06436	0.46345			
6	0.05216	0.15828	0.05275	0.40811	0.12640		
7	0.05679	0.14965	0.04862	0.44152	0.11870	0.26404	

```
================================================================================
== Exact Test of Sample Differentiation Based on Haplotype Frequencies
================================================================================
List of labels for population samples used below:
------------------------------------------------

Label     Population name
-----     ---------------
  1       sp. C
  2       landdrosia
  3       drewesii
  4       sp. A
  5       sp. B
  6       bicolor
  7       subvoce

--------------------------------------------------------------------------------
Global test of differentiation among sample:
------------------------------------------

 Non-differentiation: Exact P value = 0.00000 +- 0.00000  (6000 Markov steps done)

------------------------------------------------------------------------
Differentiation test between all pairs of samples:
------------------------------------------------
Markov chain length : 10000 steps)
```

Non-differentiation exact P values :

	1	2	3	4	5	6
2	0.00285+-0.0015					
3	0.01595+-0.0019	0.12150+-0.0122				
4	0.00205+-0.0013	0.00045+-0.0005	0.12395+-0.0178			
5	0.00025+-0.0002	0.00180+-0.0006	0.03815+-0.0045	0.00505+-0.0020		
6	0.02230+-0.0041	0.09425+-0.0124	0.46545+-0.0130	0.11830+-0.0116	0.04185+-0.0044	
7	0.00270+-0.0007	0.01650+-0.0047	0.12305+-0.0058	0.01735+-0.0022	0.00855+-0.0022	0.20000+-0.0104

Table of significant differences (significance level=0.0500):

	1	2	3	4	5	6	7
1		+	+	+	+	+	+
2	+		-	+	+	-	+
3	+	-		-	+	-	-
4	+	+	-		+	-	+
5	+	+	+	+		+	+
6	+	-	-	-	+		-
7	+	+	-	+	+	-	

Histogram of the number of significant different populations (significance level=0.0500):

1	2	3	4	5	6	7
6	4	2	4	6	2	4

Appendix 4. Map of sampled localities.