Genetic analysis of the Cape Sand Frog, *Tomopterna delalandii* (Tschudi 1838)

By

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DECLARATION

I, _____, declare that '**Genetic analysis of the Cape Sand Frog, *Tomopterna delalandii* (Tschudi 1838)"** is my own work and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

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Contents page

List of Figures

List of Tables

Abstract

Tomopterna delalandii occurs throughout the west coast, Western Cape and south coast of South Africa. This range stretches across three distinct biogeographical assemblages. Based on historical records and the fact that *Tomopterna* is a genus of cryptic frogs, it is possible that there are unknown genetic variations within the species. To investigate whether population structure is present within *T. delalandii* a mitochondrial gene and nuclear gene, 16S and Tyrosinase, was sequenced from across the range. Haplotype networks and cladograms were constructed to look at the relationship between the genetic samples. This revealed definite population structuring between samples from the western edge of the range and samples from the eastern edge of the range. It also revealed that samples taken from the northern edge of the range, while matching general *T. delalandii* tadpole morphology, are genetically different. Further study needs to be made into the identity of this new form. This study shows that by using both mitochondrial and nuclear DNA patterns can be discovered about the genetic structure of a species as well as revealing a new one.

Keywords: South Africa, *Tomopterna*, haplotypes, population structure

1. Introduction

1.1 Brief Taxonomic history

In 1838 the cape sand frog was described where it was grouped in the genus *Pyxicephalus* (Tschudi 1838). In 1841 however, a new genus of frog was described as *Tomopterna,* to which the cape sand frog now belonged (Duméril & Bibron 1841). Later scientists still referred to these frogs as belonging to the genus *Pyxicephalus* (see Günther 1858, Cope 1863). There was still some confusion to the taxonomic grouping of *Tomopterna* and in 1911 they were classified under the genus *Rana* while later classifications still referred to them as being part of the genus *Pyxicephalus* (see Hewitt 1911, Van Dijk 1978, Bowker & Bowker 1979).

Their grouping in *Pyxicephalus* led to their common name, pyxies (see Jacobsen 1978). Later on in the year the common name was proposed as sand frogs, as pyxies is only apt for true members of the genus *Pyxicephalus* (see Passmore & Carruthers 1978). It is now accepted that they belong to the family Pyxicephalidae and the sub family Cacosterninae (Zimkus & Larson 2011, Frost 2014).

The genus *Tomopterna* included species from East Asia and Madagascar until 2000, when Vences *et al.* assigned the Asian and Malagasy species to new genera, *Sphaerotheca* and *Laliostoma* respectively. *Tomopterna* is now used to describe only the African species.

Tomopterna delalandii was the first species of sand frog described (Tschudi 1838). The type locality was given as "Promontorium Bonae Spei" which is the Cape of Good Hope. Early studies refer to *T. delalandii* being found as far north as Zimbabwe and West Africa (Balinsky 1969, Broadley 1974, Jurgens 1978, Van Dijk 1978, Branch *et al.* 1988). Samples of *T.*

delalandii were also recorded as being found in Somalia, very far from its currently described range. This misidentification is mostly due to the similar morphology and colouration of the species within the genus *Tomopterna.*

1.2 *Tomopterna*

The genus *Tomopterna* currently consists of 15 species. They are *T. cryptotis, T. damarensis, T. delalandii, T. elegans, T. gallmanni, T. kachowski, T. krugerensis, T. luganga, T. marmorata, T. milletihorsini, T. monticola, T. natalensis, T. tandyii, T. tuberculosa* and *T. wambensis* (Frost 2014).

Tomopterna cryptotis is found in the central highlands of South Africa, Swaziland, Lesotho and north into Mozambique, Zimbabwe, Botswana, Namibia, Angola, Zambia and Malawi (Channing 2001). *Tomopterna damarensis* is only known from the type locality in northern Namibia, Khorixas (Dawood & Channing 2002). *Tompterna delalandii* is described below. *Tomopterna elegans* is found in north-eastern Somalia. *Tomopterna gallamanni* is found on the Laikipia Plateau of Kenya as well Maralal and Baragoi areas in the north of Kenya (Wasonga and Channing 2013). *Tomopterna kachowski* has been found in central Ethiopia into Eritrea and possibly into Sudan (Zimkus and Larson 2011). *Tomopterna krugerensis* is found from southern Angola to Namibia, through Botswana and Zimbabwe into the north eastern parts of South Africa and southern Mozambique (Channing 2001). *Tomopterna luganga* is found on the central plateau of Tanzania (Channing *et al.* 2004). *Tomopterna marmorata* is found in north eastern South Africa, Zimbabwe, Mozambique, Malawi, eastern Botswana and Zambia (Channing 2001). *Tomopterna milletihorsini* is known only from two localities in Mali (Frost 2014). *Tomopterna monticola* is known only from a single locality in Kenya (Frost 2014). *Tomopterna*

natalensis occurs from southern Mozambique southward to the Eastern Cape Province and the central highlands of South Africa (Channing 2001). *Tomopterna tandyii* is found in a broad swathe from southern Angola through Namibia and Botswana and Zimbabwe into the central highlands of South Africa and down to the south eastern coast (Channing 2001). *Tomopterna tuberculosa* is found in Namibia and Angola across the Democratic Republic of Congo and in Zimbabwe (Channing 2001). *Tomopterna wambensis* is found in north eastern Kenya and into Tanzania (Wasonga and Channing 2013).

1.3 The range of *Tomopterna delalandii*

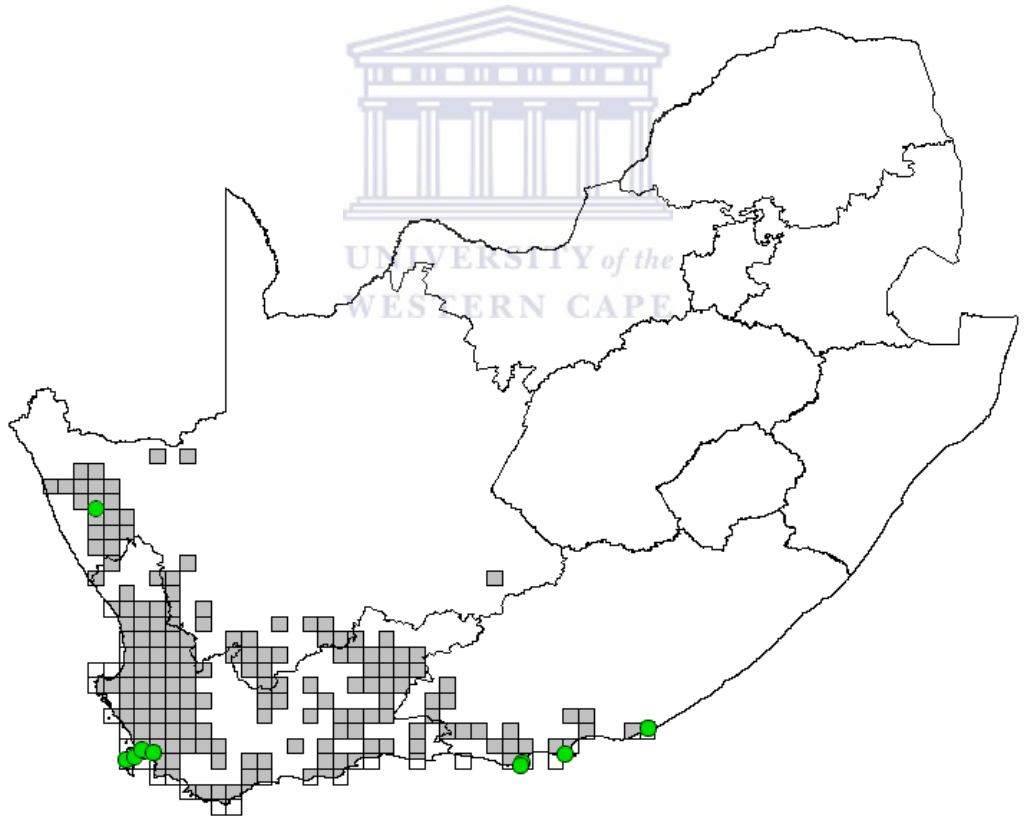

Figure 1. Distribution map for *Tomopterna delalandii* showing museum records of previously collected samples in grey squares. Study sites represented by the green circles.

The Cape Sand Frog is found in the south west of South Africa from the Cape Peninsula and Little Karoo eastward to the Eastern Cape (Port Alfred) and northward to Springbok (Van Dijk 1971, Carruthers & Carruthers 1979, Wager 1986, Channing 1988, Channing 2001) (Figure 1). Based on distribution data it appears that the species is restricted to the coastal plain and the escarpment. Even though the range seems to have a similar basic geography, the habitats in which *T. delalandii* are found cover three different biomes.

1.4 Climate and Vegetation

The Northern Cape is the driest province in South Africa with annual precipitation, along the coast less than 100 mm and as much as 400 mm at Springbok (Channing 1988, Moon & Dardis 1988, Cowling *et al.* 1999, Meadows & Hoffman 2003, Channing & Wahlberg 2011). This rainfall only occurs during the winter months, May−September (Støwer 2013). The aridity of the coast is mostly due to the Benguela current. The cool waters result in a low evaporation rate and therefore less moisture in the atmosphere (Moon & Dardis 1988). Similarly in the Western Cape, precipitation occurs mostly during the winter months (Støwer 2013). It is however more than the Northern Cape, about 500–650 mm per annum. The Western Cape has a Mediterranean climate with summer droughts and winter rains. In the east, however, rainfall drastically increases to more than 1000 mm per annum. This change in precipitation affects the vegetation found in the area. The Northern Cape range of *T. delalandii* is dominated by Succulent Karoo vegetation while the Western Cape range is dominated by Fynbos (Cowling 1990). The eastern edge of the *T. delalandii* range is dominated by Albany Thicket.

1.5 Geology

The range of *T. delalandii* within the Northern Cape falls mostly within the boundaries of the Namaqua and Natal Metamorphic Provinces. This region was prone to granite intrusions that were formed in the late Protozoic (Botha 1983). Over the years these rocks have been eroded and resulted in granite based sandy sediment (Desmet 2007). The result is a hilly landscape with flat valleys created by the eroded sandy sediment.

Along the western coast of South Africa the soil is composed of marine based sediment (Desmet 2007). This soil is composed of gray sand at the coast and red sand inland with yellow sand forming an intermediate between the two soil types. The Western Cape and Eastern Cape are mostly composed of the Cape Super Group, Cenozoic deposits and Archaen Granite. The Archaen granite is the oldest of these geological features, the intrusion of the granite resulted in the formation of gneiss. The Cape Super Group consists of various formations which are predominantly sandstone and shale based (Moon and Dardis 1988) resulting in similarly based sandy soils.

A major feature of the group is the Cape Fold Belt which stretches eastwards from the Cedarburg in the northwest through to Port Elizabeth. The Cenozoic deposits found in the Eastern Cape are the result of submergence and sediment being deposited that are of marine or fluvial origin (Johnson 1976).

The origin and underlying geology is different throughout *T. delalandii*'s range. The common factor is that in each specific area the resultant sediment is sandy, ideal for *T. delalandii*.

1.6 Biology

Tomopterna are found in sandy environment. They have flanged metatarsals that aid in burying themselves to escape predation. The burrowing also allows them to escape the heat of their environment (Rose 1926, Channing 1976). This means that throughout the year the sand needs to remain loose so that the frogs can burrow.

Although *Tomopterna* has adapted to live in dry conditions by being semi-fossorial, it still needs water to reproduce. Sand frogs commence breeding as soon as the rain falls, where they breed along river courses in shallow water and temporary pools (Balinsky 1969, Jurgens 1978, Stuart 1980). The habitat, however, still needs to be suitable for adults i.e. suitable for them to burrow during the day and feed and reproduce at night. Shallow ponds with sandy banks are ideal for the frogs. In the north this would be ephemeral river beds and natural ponds.

The main restricting factor in such an arid area will be water (Channing 1988). When looking at the Western and Eastern Cape other determining factors will effect where *T. delalandii* is found. Water is not as scarce in the Western Cape (during the rainy season) and therefore the frogs have many potential breeding sites, providing that the surrounding sediment is suitable.

Temporary factors that affect breeding are wind and temperature. If the temperature is too low it may prohibit calling, as seen in other frogs (Docherty *et al.* 1995). If the wind is too high it increases evaporation and therefore increases water loss from the frog. Wind will also interfere with the advertisement call of the frog, decreasing the chances of it attracting a mate.

Looking at the variation of the environments across the range of the cape sand frog, vast differences are notable. The same species of frog shows remarkable tolerance for fluctuating water availability, tolerating very arid conditions to areas where water is available throughout the

year. The varying amount of water available affects the vegetation types found in each region, from Succulent Karoo in the north, Fynbos in the south and Albany Thicket long the east coast.

With all the variations across the range, is the population itself genetically homogenous?

One of the factors to take into consideration when examining genetic variation is population distribution. Spatial distribution of a species within a population is often fragmented. It is important to note the gene flow between these fragments. If the gene flow is high, individuals will be similar to one another and the fragments will behave as one population. If the gene flow between the fragments is low it will result in genetically variable individuals being found within the population. Gene flow is affected by distance, amongst other things. If the distance between two fragmented patches is too far, individuals will not be able to reach the fragmented patch and interbreed which each other. This will decrease the overall gene flow of the population but increase the genetic variability. This could come into effect when looking at the population of *T. delalandii*. Approximately 1400 km separate Springbok along the coast to Port Alfred with only 950 km separating them as the crow flies.

1.7 Rationale

A study like this has the possibility to define population structuring in amphibians. This has been demonstrated by other genetic investigations.

A study was conducted looking at the genetic variation and distribution of *Amietophrynus pantherinus* (Measey & Tolley 2011). They discovered that there were genetically separated individuals across the range represented by two distinct populations. A genetic study was carried out on the spadefoot toads, and although on a larger scale, genetic variation based on geographic location was shown to be present within *Spea intermontana* (Wiens & Titus 1991). Other

molecular studies have yielded similar results, with population structure being noted in *Bufo canorus* (Shaffer *et al.* 2000) and *Hyla meridionalis* (Recuero *et al.* 2007), while others resulted in the discovery of new species (Channing *et al.* 1994, Zimkus & Larson 2011).

1.8 Aim

This study will examine genetic samples of *T. delalandii* across its range, which extends from the north west coast to the south east coast of South Africa covering very different biomes.

This study will examine genetic data based on 16S ribosomal DNA and tyrosinase exon 1 nuclear DNA. The analysis of the data will reveal whether there are any intraspecific differences. These differences will then be correlated with the sample locations to determine whether distance does play a role in the genetic variation of *T. delalandii*.

1.9 Research questions

1. With the population of *T. delalandii* covering such a variable area, is there genetic structuring within the population?

2. Does the geographic distance between the samples have an effect on the overall genetic variation?

3. Is the genetic variation, if any, similar to that found in other species of frogs?

2. Materials and Methods

2.1 Sampling

Figure 2. Map showing the localities of samples used in the study. Buff – Buffels River, CaFr – Cape St. Francis, Nord – Noordhoek, PA – Port Alfred, PE – Port Elizabth, Rond – Rondevlei, Stel – Stellenbosch, StFr –St. Francis Bay.

Samples were collected from seven sites within the range of *T. delalandii*: Noordhoek, Rondevlei, the University of the Western Cape (UWC), Stellenbosch, Port Elizabeth, St. Francis Bay and Buffels River (Figure 2). Additional tissue samples, from Cape St. Francis and Port Alfred were obtained from the Port Elizabeth Museum and the South African Institute for Aquatic Biodiversity. For the purpose of this study, samples from the west will refer to the localities Noordhoek, Rondevlei, UWC and Stellenbosch, samples from the east will refer to the localities Port Elizabeth, St. Francis Bay, Cape St. Francis and Port Alfred, while samples from north will be samples from the Buffels River. Tissue was collected from tadpoles and adults. Tadpoles were collected during the day by exploring puddles in suitable habitats. They were later

identified using a morphological key (Channing, unpublished). Frogs were collected at night by driving along roads, where they can easily be seen against the tarmac. They were also collected by investigating ponds and other suitable habitats. At ponds, male frogs were located by their calls. *Tomopterna* advertisement calls are species specific (Channing and Bogart 1996, Dawood *et al.* 2002). By comparing the vocalisations in the field to other previously recorded and confirmed calls of *T. delalandii* it ensured that the samples collected were indeed *T. delalandii.* Frogs and tadpoles were euthanized with a solution of Tricaine methanesulfonate (MS 222). In total 59 samples were collected from nine localities (Table 1).

2.2 DNA extraction from tissue samples (Modified from Hillis *et al.* 1990)

See appendix 1 for recipes.

Tissue samples were placed in labeled 1500 µl eppendorf tubes, to which 500 µl extraction buffer was added. The tissue was then macerated using sterile scissors and 15 µl of proteinase K was added to the solution. This was then left in a dry bath at 55 °C. Once the tissue was digested 500 µl of Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) was added to the eppendorf.

The solution was then shaken slightly and centrifuged for 10 min at 3 000 x g. By doing this the solution was split into two layers, the aqueous (upper) and organic (lower) phases. The nucleic acids separate out in the aqueous phase, while proteins separate into the organic phase. The aqueous layer was then carefully removed using a micropipette with a wide bore tip and transferred to a new eppendorf. Care was taken not to disturb the interface and draw any proteins into the aqueous solution. A second, more stringent separation was carried out by adding 500 µl of Chloroform: Isoamyl alcohol (24: 1) to the previous aqueous layer. The eppendorf was gently shaken and centrifuged for a further 10 min at 3 000 x g. The aqueous layer was removed and

transferred to a new eppendorf. To force the DNA to precipitate 45 µl of 3 M Sodium Acetate and 650µl of ice cold absolute ethanol was added. The solution was then placed in a freezer overnight to allow the DNA to precipitate.

The following day, the samples were removed from the freezer, placed in a centrifuge and spun at 12 000 x g for 10 min. The eppendorf was then slowly emptied of excess liquid leaving behind the precipitated DNA pellet. The tube was then left open to dry at room temperature until all the excess liquid evaporated. The pellet was then suspended in 50 µl TE. To samples that had a small amount of tissue initially, 30 µl TE was added.

In order to determine the concentration of DNA in the solution, it was quantified, following the instructions provided by Invitrogen for a Qubit fluorometer. The solution was then diluted with sterile water to attain a DNA template of 2 ng/ µl.

2.3 Polymerase Chain Reaction (Modified from Hillis *et al.* 1990)

PCR tubes were prepared with 12.5 µl Kapa ready mix , 1 µl of 16Sar-L primer (10nm), 1 µl 16Sbr-H primer (10 nm), 6.5 µl purified water and 4 µl of diluted template DNA (2ng/ µl). A negative control was made by following the same recipe except replacing the DNA with purified water. This process was repeated using primers for nuclear genes, tyrosinase exon 1C (Tyr C) and tyrosinase exon 1G (Tyr G).

16SA: 5’ – CGC CTG TTT ATC AAA AAC AT – 3’ (Palumbi *et al.* 1991)

16SB: 5’ – CCG GTC TGA ACT CAG ATC ACG T – 3’ (Palumbi *et al.* 1991)

Tyr C: 5’ – GGC AGA GGA WCR TGC CAA GAT GT – 3’ (Bossuyt and Milinkovitch 2000)

Tyr G: 5’ – TGC TGG CRT CTC TCC ART CCC A – 3’ (Bossuyt and Milinkovitch 2000)

The samples were then placed in a Techne TC-512 PCR machine and run in 3 stages. For 16S:

Stage 1: 95 °C for 1min

Stage 2: 95 °C for 10 s, 51 °C for 10 s, 72 °C for 1 s (Stage 2 was repeated 35 times)

Stage 3: hold at 10 °C

For tyrosinase exon 1:

Stage 1: 95 °C for 1 min

Stage 2: 95 °C for 10 s, 55 °C for 10 s, 72 °C for 1 s (Stage 2 was repeated 35 times)

Stage 3: hold at 10 °C

To visualize the amplified PCR product a 0.8 % agarose gel was prepared. Prior to the gel setting 2 µl of ethidium bromide was added. The ethidium bromide inserts into the DNA strand, and makes it visible under UV light. The PCR product was then visualized using a Spectroline ultraviolet transilluminator and sent to the Central Analytical Facility at Stellenbosch University for clean up and sequencing.

2.4 Analysis

2.4.1 16S

Sequences received from the Central Analytical Facility were opened and viewed in BioEdit (Hall 2007). Each sequence and associated chromatogram was individually examined. At sites where no definite base was given, they were replaced with the appropriate base based on that sequence's chromatogram. The dataset was then combined with sequences from GenBank.

Sequences were then aligned in Clustal X v2.1 (Larkin *et al.* 2007) where a complete alignment was run in multiple alignment mode. Sequences were subsequently trimmed using BioEdit (Hall 2007) to ensure equal sequence length. These were then used to create a haplotype network in TCS v1.21 (Clement *et al.*2000). Gaps were selected as missing data.

Phylogenetic analyses were carried out using Bayesian Inference (MrBayes v3.0b4, Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). To determine which model to use in Mr Bayes, jModelTest 0.1.1 was used (Dariba *et al.* 2012). The Bayesian analysis was then run using the GTR + gamma model (nst=6, rates = gamma). A search was run using the Markov Chain Monte Carlo algorithm. The search contained four Markov chains, one cold and three heated, which was run for 9 000 000 generations with trees saved every 1000 generations. The first 2250 trees were discarded as burnin and a consensus tree was constructed from the remaining sampled trees. For these analyses, *Pyxicephalus adspersus* was selected as the out group as amphibian phylogeny shows that they are grouped in the same clade to *Tomopterna* yet are phylogenetically basal (van der Meijden *et al.* 2005, Pyron & Wiens 2011).

From the dataset used to create the consensus tree, a haplotype list was created in MacClade v4.05 (Maddison & Maddison 2002). This process combined sequences that have exactly the same order of bases into one sequence, turning 40 sequences into nine distinct haplotypes. The haplotype dataset was transferred into Arlequin v3.5 (Excoffier & Lischer 2010) where details such as locality and grouping, Western or Eastern Cape, were attached to each haplotype. In Arlequin v3.5 an analysis of molecular variance (AMOVA) was carried out to test variability between sequences within a group and between groups.

Neutrality tests were also conducted using Arlequin v3.5 (Excoffier & Lischer 2010). Selective neutrality was measured using Fu's Fs-test (Fu 1997) and Tajima's *D* statistic (Tajima 1989), 10 000 samples were simulated. F statistics were calculated to show population structure.

To support the population structure produced by the AMOVA analysis a Mantel test was run. The test correlates geographic distance and genetic variation. An uncorrected p matrix of genetic variation was generated in PAUP* v4.0b10 (Swofford 2003). ArcView GIS 3.3 was used to generate a geographic distance matrix. These two matrices were combined in MANTEL v1.19 (Cavalcanti 2008) and the analysis run.

2.4.2 Tyrosinase exon 1

Nuclear DNA is diploid unlike the haploid mitochondrial DNA. This is important when analysing the sequence and chromatogram. When looking at a chromatogram of nuclear DNA, polymorphic positions would appear as two peaks of similar height, each allele carrying a different base. This means that ambiguous bases cannot be resolved by selecting one base in favour of another based on the chromatogram as done with mitochondrial DNA. This is because each base comes from one of the parents. To solve the ambiguity, PHASE v 2.1 (Stephens *et al.* 2001, Stephens & Scheet 2005) estimates the haplotypes for both parents from the single sequence. PHASE input files are generated by SEQPHASE (Flot 2010). Output files from PHASE are then converted back into a more compatible format using SEQPHASE. This returns all the possible haplotypes with their respective probability. From this, the two sequences with the highest probability are selected, representing each parent.

Both sequences, for each sample, are then aligned and trimmed as with the 16S methodology. Haplotype networks were then constructed using the tyrosinase exon1 data set.

For the Bayesian analysis the GTR + gamma model was also indicated to be the best model. The Markov Chain Monte Carlo analysis was run for 20 000 000 generations with trees saved every 1000 generations. The first 5000 trees were discarded as burnin and a consensus tree was constructed from the remaining trees.

The analyses run for 16S were then repeated using the tyrosinase exon 1 data set.

2.4.3 Comparative Analysis

Genetic variation is expected within a species. Differences between samples from within a species would suggest population structuring. To compare whether the genetic variation within *T. delalandii* is low enough to suggest only one population, sequence data of the 16S mitochondrial gene of other frogs were taken and compared to the 16S results of *T. delalandii*. The species used for the comparative analysis were *Amietophrynus rangeri* and *A. regularis, Phrynobatrachus parvulus, Ptychadena anchietae* and (Table 2). Species were selected on whether they share a similar range with *T. delalandii*. If species were not from the same area, they were selected on whether the distance between the furthest points of their range is similar or more than that of *T. delalandii*.

3. Results

3.1 Sequences

The data set for *T. delalandii* consists of 59 individuals. For the 16S gene, 53 of those samples sequenced successfully. The data set for tyrosinase exon 1, 23 samples sequenced successfully (resulting in 46 sequences overall due to the polymorphic base pairs).The 16S dataset was supplemented with six haplotypes from GenBank. Sequences received from the Central Analytical Facility had varying sequence lengths, with an average of 572 base pairs per sequence for 16S and 552 base pairs per sequence for tyrosinase exon 1. The sequences from GenBank consisted of sequences that had 444 base pairs to 2408 base pairs (Table 1.1). To prepare them for analysis the sequences were trimmed to 344 base pairs for 16S and 528 base pairs for the tyrosinase exon 1 sequences. This was done to trim bases from the front and ends of the sequences and to ensure uniformity between the sequences.

3.2 Haplotype network

Haplotype networks show the relationships between sequences. Closely connected haplotypes mean that there a few base pair changes between them. Haplotypes that are further apart have many base pair changes between them. If two clusters of haplotypes are far apart it would suggest that there is some population structure present. When a haplotype network is combined with geographic locations it also visually shows whether isolation by distance is occurring within the species.

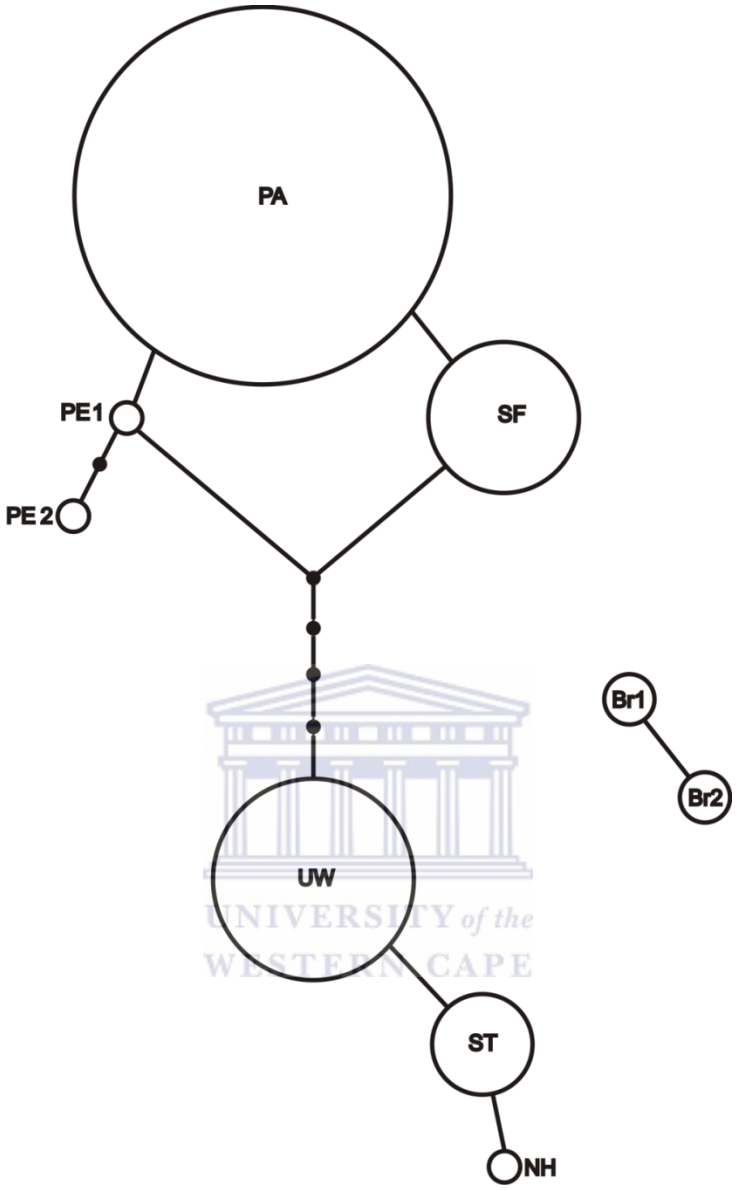

Figure 3. Haplotype network of the 16S gene for *Tomopterna delalandii*. Haplotypes represented by abbreviations, including other localities that share the haplotype, are BR1-Buffels River (n=2); BR2-Buffels River (n=2); NH1-Noordhoek (n=2), and Stellenbosch (n=2);); NH2-Noordhoek (n=1); PA-Port Alfred (n=21); PE1-Port Elizabeth (n=1); PE2-Port Elizabeth (n=1); SF-St. Francis Bay (n=6); UW-University of the Western Cape (n=2), Rondevlei (n=1) and Stellenbosch (n=5).

The haplotype network for 16S (Figure 3) shows a definite clustering of haplotypes in three distinct groups. Haplotypes PA, PE1, PE2 and SF are found in the eastern edge of the population range, around Port Elizabeth, while haplotypes UW, ST and NH are found in the western edge of

the range, around Cape Town, approximately 790 km away. The results show population structuring within the species.

Haplotypes BR1 and BR2 are not connected to the rest of the network under the 95 % connection limit. Haplotype BR1 is shared by two individuals and haplotype BR2 is shared by two individuals. These specimens were found in the Buffels River in the northern part of *T. delalandii*'s range. Approximately 450 km separate the northern sampling site from the western sites. That is 350 km shorter than the distance separating the western and eastern groupings. It would be expected that individuals closer to one another would be more similar to each other. The rate at which individuals will be able to interbreed will be higher therefore an increase in the gene flow (Amos & Harwood 1998).

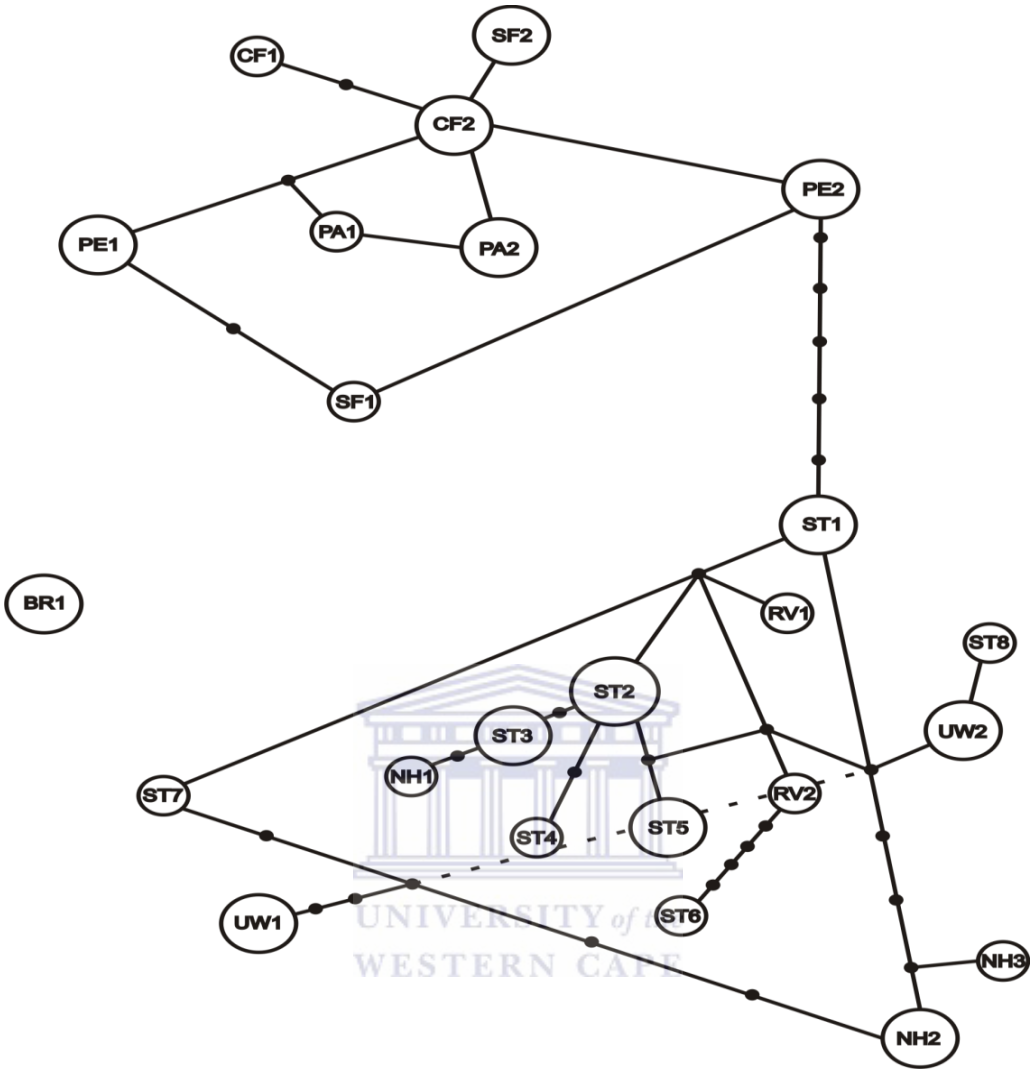

Figure 4. Haplotype network for the tyrosinase exon 1 nuclear gene for *Tomopterna delalandii.* Haplotypes represented by abbreviations, including other localities that share the haplotype, are BR-Buffels River (n=2); CF1-Cape St. Francis (n=1); CF2-Cape St. Francis (n=2); NH1-Noordhoek (n=1); NH2-Noordhoek (n=1); NH3-Noordhoek (n=1);PA1-Port Alfred (n=1); PA2-Port Alfred (n=2); PE1-Port Elizabeth (n=2); PE2-Port Elizabeth (n=2); RV1-Rondevlei (n=1); RV2-Rondevlei (n=1); SF1-St. Francis Bay (n=1);; SF2-St. Francis Bay (n=2); ST1-Stellenbosch (n=2); ST2-Stellenbosch (n=4); ST3-Stellenbosch (n=2); ST4-Stellenbosch (n=1); ST5-Stellenbosch (n=1); ST6-Stellenbosch (n=1); ST7-Stellenbosch (n=1); ST8-Stellenbosch (n=1); UW1-University of the Western Cape (n=2); UW2-University of the Western Cape (n=2)

In the tyrosinase exon 1 network (Figure 4) many haplotypes are present. The haplotypes are grouped into two distinct regions. The first grouping of haplotypes is from the western edge of the species range, Stellenbosch, UWC, Noordhoek and Rondevlei. Within the western grouping, there is high variability between the haplotypes themselves, with as many as 13 base pair

changes between two haplotypes. The other grouping of the haplotypes is from the eastern edge of the range, Port Elizabeth, Port Alfred, Cape St. Francis and St. Francis Bay. In comparison to the previous group, the variation between the haplotypes is low. Haplotype PE2 is only 6 base pair changes away from haplotype ST1. Haplotype BR1 is from the northern part of the range, and is not connected to the other haplotypes. The results question the validity of the samples from the Buffels River as it differs in the mitochondrial gene and the nuclear gene. Based on the morphological key it was clear that the tadpoles collected from the Northern Cape were in fact *Tomopterna*. Based on the high variation between the samples, it is questionable whether they are the same species.

When comparing the uncorrected p values between localities, values varied from 0.0 % to 3.5 %. Samples from the Buffels River had higher p values when compared to the rest of the localities, 2.0 to 3.5 %. The Buffels River samples were then removed from the dataset and compared to other sequences on GenBank using the Nucleotide BLAST option. The result was that the sequences had a 97 % maximum identity to sequences from *T. tandyii*, *T. damarensis*, *T. elegans*, *T. cryptotis*, and *T. delalandii*. Based on the lack of connectivity in the haplotype network and the high variability shown using uncorrected p values data from the Northern Cape has been omitted from the AMOVA and Mantel analysis.

3.3 Cladograms

For the 16S cladogram (Figure 5) there is strong support for two separate clades, one from the western edge of the range and the other from the eastern edge of the range. There is a clade support of 51 % grouping the samples from the western edge. There is however a stronger support (83 %) for the grouping of the samples from the eastern edge of the range. There are also outliers GB 1 and 2, which are most likely of another species of *Tomopterna*.

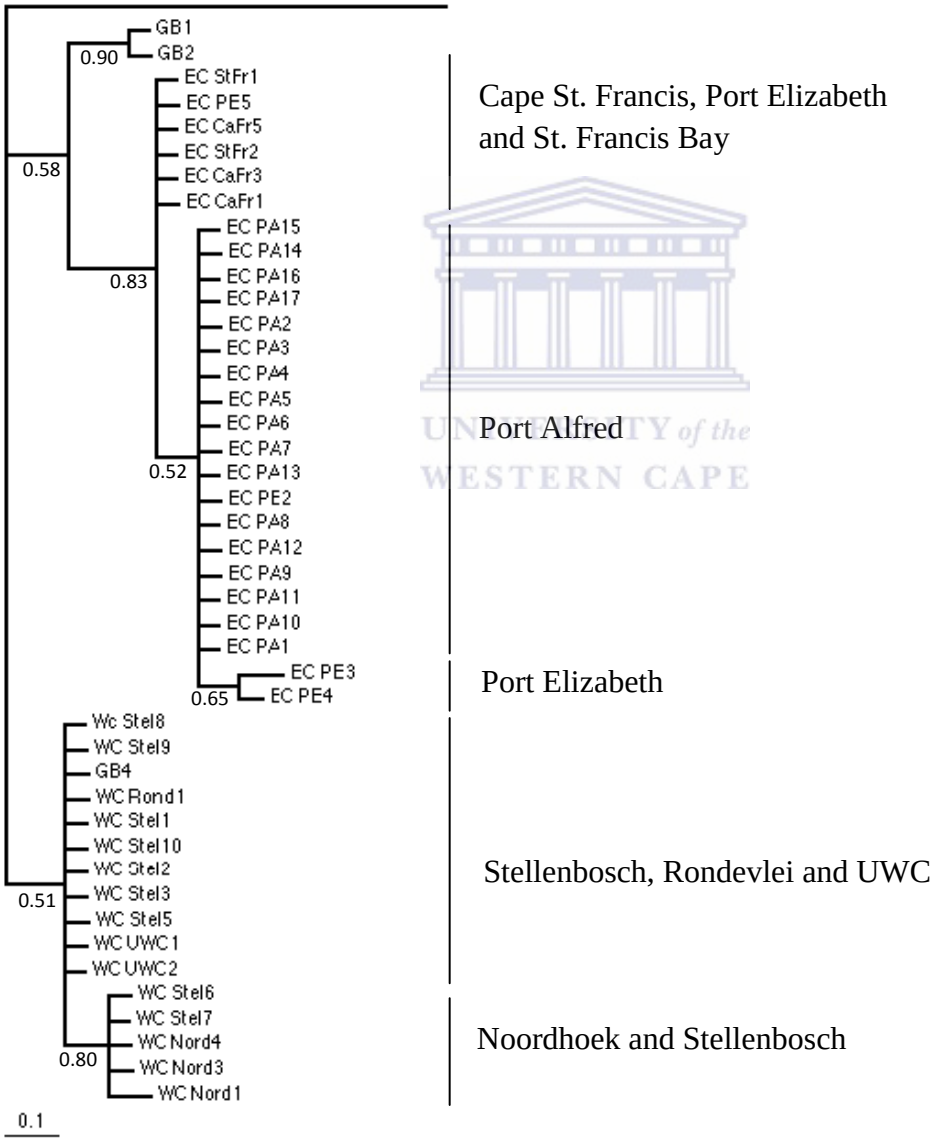

Figure 5. Cladogram generated by MrBayes for the 16S mitochondrial gene

For the tyrosinase exon 1 cladogram (Figure 6) the eastern edge grouping is clearly visible in the top half of the cladogram with 100 % support of the tree. The eastern edge samples are all definitively grouped together with a clade credibility of 97 %. The northern samples are also visible and are most closely related to samples from the western range.

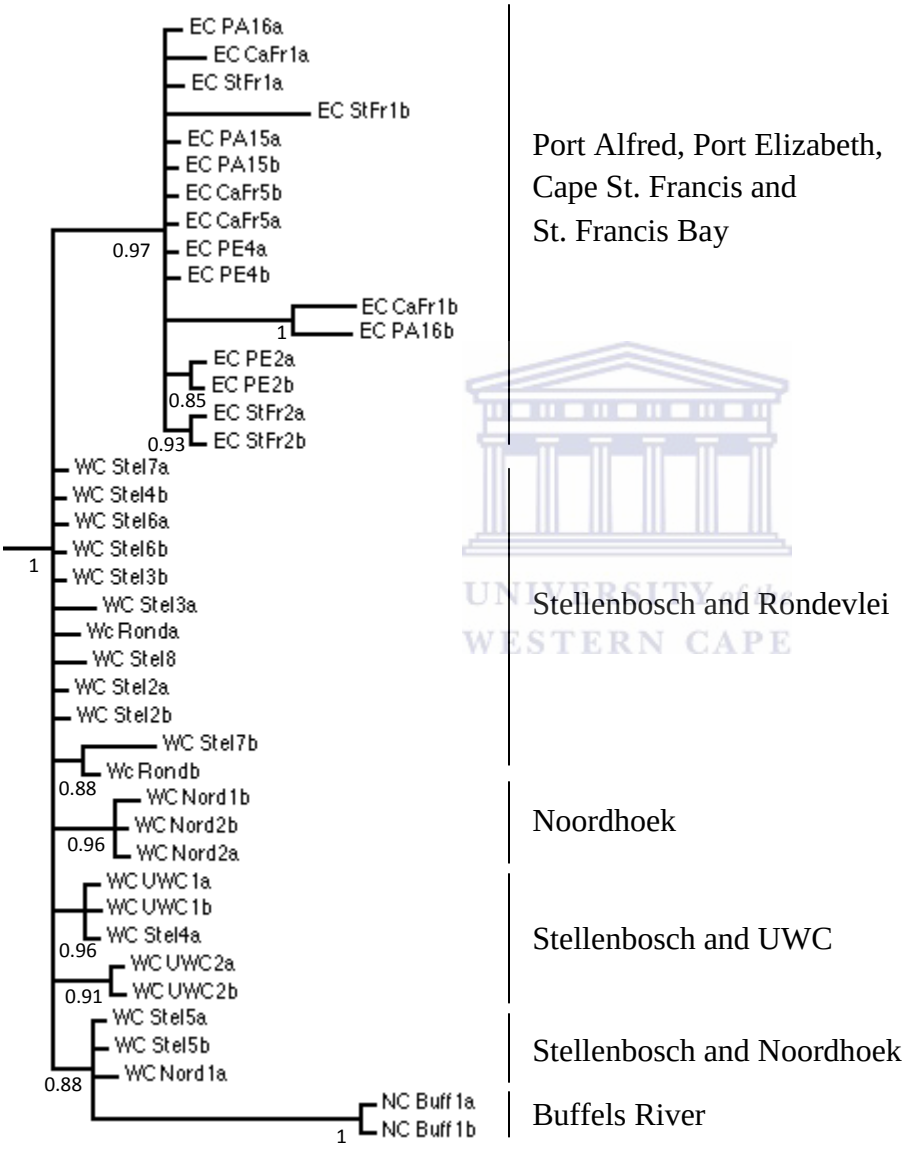

Figure 6. Cladogram generated by MrBayes for the tyrosinase exon 1 nuclear gene

3.4 Analysis of Molecular Variance

Table 3: Analysis of Molecular Variance between the Western Cape and Eastern Cape grouping of haplotype samples of *Tomopterna delalandii*, giving the sum of squares, variance components and percentage variation between the haplotypes.

	16S				Tyrosinase exon 1			
Source of variation	d.f.	Sum of squares	Variance components	Percentage variation	d.f.	Sum of squares	Variance components	Percentage variation
Among groups	1	53.248	2.6642 va	83.51	1	84.045	31.3072 va	22.88
Among populations within groups	6	7.535	0.26627 vb	8.34	6	81.367	0.75828 vb	5.54
Within populations	33	8.583	0.26010 vc	8.15	36	352.542	9.79282 vc	71.58
F_{SC}			0.50586				0.07187	
F_{ST}			0.91853				0.28425	
F_{CT}			0.83514				0.22882	

The AMOVA analysis (Table 3) had haplotypes that were grouped according to their geography. There was a difference of 8.34 % among the 16S samples. This is to be expected as there are no major physical boundaries within each group preventing organisms from breeding with each other. Each group also has similar climate with localities being no further than 43.2 km apart in the western grouping and 204 km in the eastern grouping. This low genetic variation between populations is mirrored by the low variation within the populations themselves, 8.15 %. The low variation is due to the fact that samples from each locality were found close to one another, less

than 100 m apart and genetic variation is not expected to change over such a small distance. A high variation was noted between the group in the eastern edge of the range and the group in the western edge of the range 83.51 %.

Fixation Indices are used to examine levels of variation within a population. These variations, if any, are the result of population structure. The variance between samples in a group (F_{SC}) was 0.505. While not as low as expected, this median value shows that there is some variation within the groups. This value is not high enough to warrant the formation of another group. The variance between groups (F_{CT}) is high at 0.84. This means that group 1 from the western end of the range is significantly different to group 2 on the eastern edge of the range. This supports the AMOVA results. The variation between all sites (F_{ST}) was 0.92. This high fixation index suggests that there is definite population structuring within *T. delalandii*. The fact that it is so high suggests very little interbreeding occurs between the two groups.

AMOVA results are more conservative when looking tyrosinase exon 1. The variation between groups is low (22.88 %). This is expected as a high variation within nuclear DNA would suggest that two different organisms were used in the analysis. There was however a low variation between populations within a group (5.5 %) which show that even though there is variation within the groups it is not significant. The variation within a population was however relatively high (72.58 %). This is most likely due to the base variation of haplotype seven compared to other samples from the same area. Similarly for haplotypes 12 and 18 which both come from Stellenbosch.

3.5 Test for neutrality and diversity

Table 4: Diversity and Neutrality test results between the 16S gene and tyrosinase exon 1 gene for *T. delalandii*.

	16S		Tyrosinase exon 1	
	Western Cape	Eastern Cape	Western Cape	Eastern Cape
Gene Diversity	0.6917 ± 0.0864	0.4701 ± 0.0962	1 ± 0.0137	0.9957 ± 0.0153
Nucleotide Diversity	0.003596 ± 0.002693	0.001894 ± 0.001686	0.016455 ± 0.008807	0.061077 ± 0.030905
Tajima's D	-0.08238	-0.63229	-1.17392	-1.12723
Tajima's D p value	0.4136	0.3051	0.1138	0.1138
Fu's Fs	0.21471	-0.7048	-15.9874	-3.7929
Fs p value	0.5493	0.2608	0	0.0583

Together with the AMOVA analysis, diversity and neutrality tests were carried out. The western group has high gene diversity at 0.69 ± 0.09. This high gene diversity value would explain the F_{SC} value being higher than expected. Gene diversity looks at the loci between two sequences, while nucleotide diversity focuses on the population as a whole. The nucleotide diversity for the western group is 0.004 ± 0.003 (Table 4).

When looking at the eastern grouping of populations, it is evident that they are more similar to each other than the western group due to having lower gene diversity (0.47 ± 0.09). The nucleotide diversity is also low, 0.002 ± 0.002. The low diversity values indicate that samples from the eastern grouping, St. Francis bay, Cape St. Francis, Port Elizabeth and Port Alfred, are more similar to each other than when compared to the western grouping.

Neutrality tests allow insight as to whether population is growing and evolving randomly or if there is another non-random process taking place. The Tajima's D value of -0.08 suggests that the population is either going through purifying selection or expansion. This is the same for the

grouping from the east, Tajimas D = -0.63. Although negative, these values are too small to be considered significant, similarly with Fu's neutrality test.

The western grouping has positive value of 0.21. This would suggest that the western population has undergone a bottleneck. The eastern grouping alternatively has a negative value of -0.71. This would suggest that the population is expanding. The p value, however, does not support either Fs value.

3.6 Mantel Analysis

Table 5: Results of Mantel test for *T. delalandii* and four other species with accompanying t and p values

	r	t	p	Source
A. rangeri	0.308	1.572	0.942	Cunningham & Cherry (2000)
P. parvulus	-0.012	-0.127	0.449	Siow *et al.* (2009), Zimkus & Schick (2010(
P. anchietae	0.515	3.761	0.999	Vences *et al.* (2004), Measey *et al.* (2007), Siow *et al.* (2009)
A. regularis	-0.106	-1.023	0.153	Vasconcelos *et al*. (2010)
T. delalandii	0.935	29.018	1	This study

While the r values suggest that there is at least a certain amount of correlation between geographic distance and genetic variation (Table 5), the lack of a supporting p value reveals no significant relationship between the two variables being investigated.

3.7 Comparative results

As seen from the previous results the genetic variation of *T. delalandii* differs significantly with geographical distance. Comparative analyses were carried out to test if this level of variation is found in the selected species of frogs.

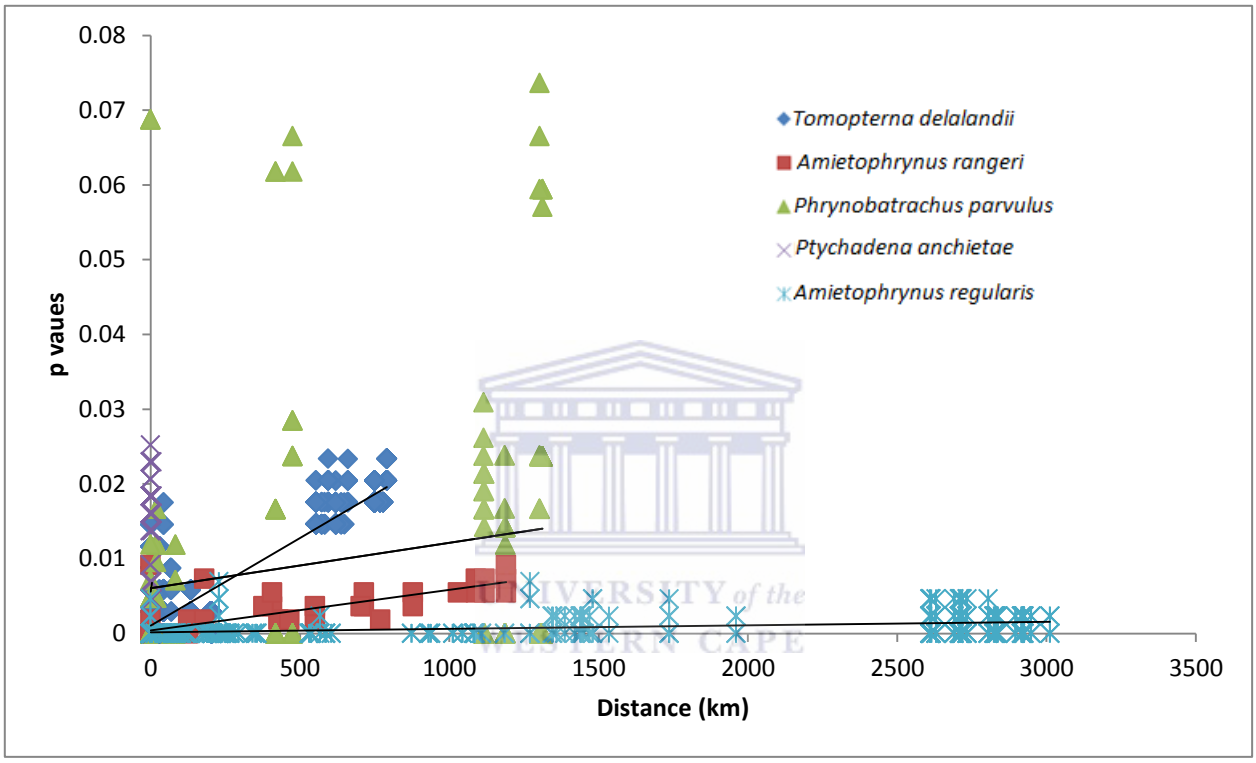

Figure 7. Graph showing the relationship between genetic distance and geographic distance in various species of frogs.

To ensure that each sequence used was of the same area of the gene they were aligned and trimmed. This resulted in each sequences for each specific species having the same number of base pairs. For *A. rangeri* seven sequences were collected and they were trimmed to a 546 base pairs. *Amietophrynus regularis* had 22 available on GenBank and each trimmed to 435 base pairs while *P. parvulus* had 16 sequences available and each sequence was trimmed to 424 base pairs. *Ptychadena anchietae* had 12 sequences available and they were trimmed to 437 base pairs.

The graph (Figure 7) was then created using the genetic variation from PAUP* v4.0b10 (Swofford 2003) and the geographic data from ESRI.

The figure shows that generally as distance increases so does the genetic variation. What is interesting to note is the fact that *T. delalandii* has a faster rate of change over other species that have a much larger range. This is especially true when being compared to *A. rangeri*, a toad also from South Africa. This species has a larger range than *T. delalandii* yet it also overlaps with the cape sand frog's range. The graph shows that the peak of variation in *A. rangeri* does not correlate with the peak of *T. delalandii*.

Amietophrynus regularis has the lowest genetic variation compared to all the species yet it has the greatest distance between points. *Phrynobatrachus parvulus* has the highest initial variation but as the distance increases the genetic variation does increase slowly. This is most likely due to very isolated populations close to one another. The same can be seen in *P. anchietae*, but more data points will have to be collected before conclusions can be drawn from that species.

4. Discussion

4.1 Population Structuring: East vs. West

This study shows that when looking at the mitochondrial DNA as well as the nuclear DNA, there is definite population structuring within *Tomopterna delalandii*. Both the cladogram and the networks (Figures 3-6, 8) show that the western edge of the population is different from the eastern edge within South Africa. This is supported by the AMOVA results. When looking at these two geographic locations of the eastern and western groupings there are many possible reasons for a lack of gene flow.

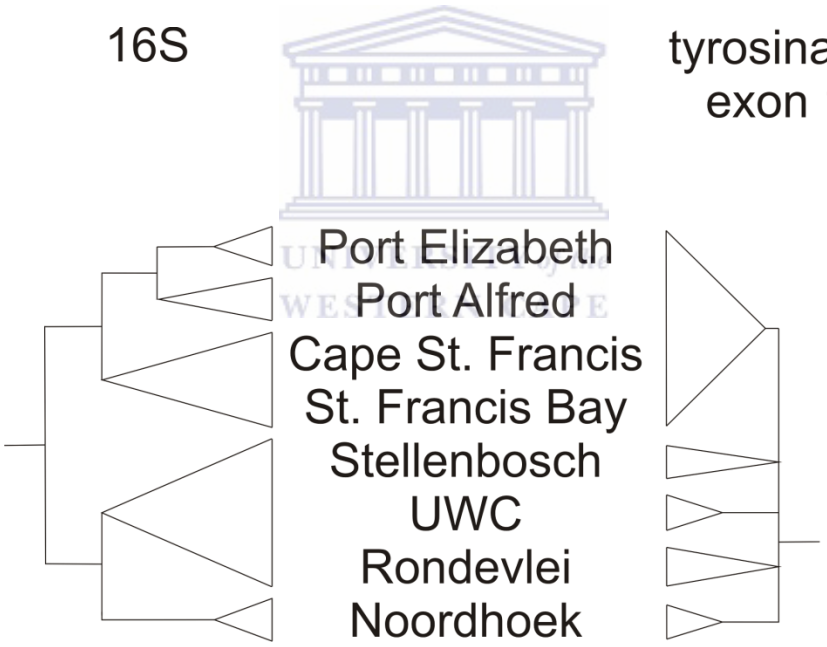

Figure 8. A comparison of the 16S and tyrosinase exon 1 trees for *Tomopterna delalandii*.

Rainfall is usually associated with amphibian distribution. Along the south coast of South Africa there are two main rainfall patterns. The western edge of the range falls under the winter rainfall zone and the eastern edge falls in the southern rainfall zone. The fact that the rains fall at different times however could be a reason as to why the frog's breeding times are not concurrent.

There are also no major physical barriers between the two areas. While the mountain ranges to the north are possible barriers to north-south movement, there is no physical barrier preventing them from moving in an east-west direction. This is corroborated by the neutrality tests which show that no isolation is occurring due to distance. It would then be beneficial to collect samples of *T. delalandii* from intermediated distances between the two populations. Previous studies, which showed similar patterns of population structure, suggest that although there are no current boundaries between the two clades, barriers that existed in the past may have influenced present day patterns (Tolley *et al*. 2006, Price *et al.* 2007, Swart *et al*. 2007, Tolley *et al.* 2010). Climate change events during the late Pliocene and Pleistocene altered and changed the landscape of the South African coast. Sea level changes have exposed and inundated much of the continental shelf and crustal upliftment resulted in altering altitudes and different rain shadows. These changes most likely presented barriers to *T. delalandii*. Another possibility is that is that with the overall climate change, the winter rainfall zone became fragmented resulting in isolated dry areas (Swartz *et al.* 2007). This would have prevented individuals from dispersing.

When looking at the data as a whole and noting which localities have genetic differences, each population (western, eastern and northern) falls within a different biogeographical assemblage (Alexander *et al.* 2004). Samples from the western edge of the population range fall within the South Western Cape assemblage. This assemblage belongs to a greater Winter/ Transitional District. This greater district does still not include the population found on the eastern side of *T. delalandii*'s range. The population here falls under the Eastern Escarpment/ Coastal District. It appears that there exists or existed a boundary between these two districts that is driving the population structuring present in *T. delalandii*. The authors have also tied in their descriptions of these biogeographical units with similarities found in ecological vegetation units (Alexander *et*

al. 2004). This suggests that there might also be ecological processes that are driving the changes seen in the population structure (Alexander *et al.* 2004).

The *Tomopterna* frogs from the northern edge of the range fall within a totally different assemblage, the Namaqualand Assemblage. This assemblage has a low species richness (Alexander *et al.* 2000). This means that the discovery of a new species within this assemblage is in itself important. Similarly as above, the fact that it is found within a separate assemblage implies that ecological, and even allopatric, factors have been in play. Unlike in the case above, where the population of *T. delalandii* is structured but still in essence a single species, these factors have resulted in a separate species.

Dispersal evidently was hindered when moving north. Samples from the Buffels River in the Northern Cape were initially assumed to be *Tomopterna* based on the tadpole morphology. The sample's locality, near previously recorded sites of *T. delalandii,* led to the decision that the samples were also *T. delalandii*. When analyzing the genes though, the specimens from the Buffels River were significantly different to the others in both the 16S mitochondrial DNA and the tyrosinase exon 1 nucleic DNA.

In terms of geographic location the nearest *Tomopterna* species is *T. tandyii.* To rule out the possibility that the tadpoles were washed down tributaries from another locality (Channing 1976), the river and all its tributaries were traced in Google Earth and overlaid with a distribution map of *T. tandyii*. This revealed that there was no overlap between the river and range *T. tandyii*. Based on the high p value compared to the rest of the species and the locality of the specimen, the samples collected from the Buffels River are not *T. delalandii*.

Further research needs to be done to ascertain whether this is a new species of *Tomopterna*. With the genetic differences as presented, there is definitely an undescribed species of *Tomopterna* in the Northern Cape. This then raises the question of whether preciously record specimens of *Tomopterna* in the Northern Cape were in fact *T. delalandii* or this new species of *Tomopterna*.

Based on the Mantel analysis, it cannot be said conclusively that there is a correlation between distance and genetic variation, but when looking at *T. delalandii* compared to other species of frog, the species seems to show more variation within a smaller region compared to other species (Figure 7).

4.2 A New Species

While the eastern grouping is slightly different from the genetically defined *T. delalandii*, samples from the Buffels River are clearly different. The haplotype networks show no immediate connection the samples from this locality. Uncorrected p values showed a 2.0 – 3.5 % difference between the Buffels River samples and *T. delalandii* from all other localities. A BLAST analysis showed that the Buffels River sequences were 3 % different to sequences of *T. tandyii*, *T. damarensis*, *T. elegans*, *T. cryptotis* and *T. delalandii* in GenBank.

An uncorrected p matrix was generated using known sequences of only *T. delalandii* from GenBank to double check inter species variation. These sequences only had a variation of 0.0–0.75 %. Channing *et al.* (2004) showed a 2.9–3.2 % sequence divergence between *T. delalandii* and *T. cryptotis* and a 1.4–1.6 % sequence divergence between *T. delalandii* and *T. tandyii*. This is similar to the divergence shown by Dawood and Channing (2002), 3.5 % between *T. delalandii* and *T. cryptotis* and 1.6 % between *T. delalandii* and *T. tandyii*. When looking at the

variation between *Tomopterna* species it is clear that a variation of 2–3.5 % is sufficient enough to distinguish two species from each other.

While understandably these previous studies had more than just genetic information as the basis for the description of a new species, the fact that the difference in the Buffels river samples is larger than previously described margins cannot be ignored.

4.3 A Speciation Event

The data shows that the eastern half of the population is distinctly different from the western half. Based on the results it appears as if the eastern grouping is going through a speciation event. Looking at the haplotype networks it is evident that no haplotypes, mitochondrial or nuclear, are shared between the two groupings. So while there are no physical barriers preventing the two from interbreeding, there appears to be no interbreeding between them anyway. This would mean that the populations themselves are allowed to diversify and therefore evolve separately from one another. The cladograms suggest that the eastern grouping is younger and therefore the most recent. This adds to the notion that this group in particular is going through the speciation event rather than western grouping.

This would suggest that the eastern grouping is not only different from the western grouping but also that the group has diverged and is becoming a new species.

4.4 Conclusion

The overall pattern in *T. delalandii* shows that population structuring is present. This is to be expected as the range that they cover is variable. It appears that the distance between the samples does have an effect on the overall genetic variation, however negligible the mantel analysis

suggests. This would be better supported if more samples were collected from along the south coast. A new study along this west-east axis would provide a better understanding of how individuals are interacting between the western and eastern edge of the range. It would also show whether there are any definite barriers, ancient or present, preventing individuals from migrating.

It appears that the variation found within *T. delalandii* is uniquely high. This supports the population structuring found in the haplotype networks and the cladograms. This shows that the species needs more study, especially samples from Noordhoek and the eastern edge of the range (Port Alfred and Port Elizabeth). The Noordhoek samples corroborates that high variation can be found within a short geographic distance.

16S and Tyrosinase exon 1 proved to be successful for this kind of genetic study. The amplified barcode region that these primers produced was successful in showing variations within a species. This is significant when trying to identify if there is any population structuring in a given population.

Within the species *T. delalandii*, as presently understood, there appears to be at least two species of frog and definite population structuring. Further studies need to be carried out in the northern edge of the range to ascertain fully whether the frogs identified as *T. delalandii* are in fact a different species or whether *T. delalandii* and this different species share a range. If they are a new species, population boundaries need to be re-examined.

Based on the above further research questions would be:

Is there further evidence to support the differences found in the genetics between the eastern and western samples of *T. delalandii*?

What does the population of *T. delalandii* look like between Stellenbosch and Cape St. Francis? Are there any breeding gaps? Are there any distinct genetic boundaries?

Are there morphological and acoustic differences that would support the finding of a new species from the Buffels River?

Table 1: Specimens used for sequencing listing field numbers, locality, district, province, geographic co-ordinates and tissue source.

Field number	Locality	District	Province	Co-ordinates	Source of Tissue
AC 3222	Stellenbosch Air Field	Cape Town	Western Cape	33°58'56.34"S, 18°49'12.91"E	adult thigh
AC 3223	Stellenbosch Air Field	Cape Town	Western Cape	33°58'56.34"S, 18°49'12.91"E	adult thigh
AC 3224	Stellenbosch Air Field	Cape Town	Western Cape	33°58'40.74"S, 18°49'6.71"E	adult thigh
AC 3225	Rondevlei	Cape Town	Western Cape	34° 3'30.29"S, 18°30'15.43"E	adult thigh
AC 3226	UWC	Cape Town	Western Cape	33°55'54.07"S, 18°37'24.88"E	adult thigh
AC 3227	UWC	Cape Town	Western Cape	33°55'54.07"S, 18°37'24.88"E	adult thigh
AC 3228	Stellenbosch Air Field	Cape Town	Western Cape	33°58'56.34"S, 18°49'12.91"E	adult thigh
AC 3229	Stellenbosch Air Field	Cape Town	Western Cape	33°58'56.34"S, 18°49'12.91"E	adult thigh
AC 3230	Stellenbosch Air Field	Cape Town	Western Cape	33°58'56.34"S, 18°49'12.91"E	adult thigh
AC 3231	Stellenbosch Air Field	Cape Town	Western Cape	33°58'56.34"S, 18°49'12.91"E	adult thigh
AC 3232	Noordhoek	Cape Town	Western Cape	34° 5'49.07"S, 18°22'24.34"E	adult thigh
AC 3233	Noordhoek	Cape Town	Western Cape	34° 5'49.07"S, 18°22'24.34"E	adult thigh
AC 3234	Noordhoek	Cape Town	Western Cape	34° 5'49.07"S, 18°22'24.34"E	adult thigh
AC 3235	Lovemore Park	Port Elizabeth	Eastern Cape	34°0'6" S, 25°31'40" E	adult thigh
AC 3236	Lovemore Park	Port Elizabeth	Eastern Cape	34°0'6" S, 25°31'40" E	adult thigh
AC 3237	Lovemore Park	Port Elizabeth	Eastern Cape	34°0'6" S, 25°31'40" E	adult thigh
AC 3238	Lovemore Park	Port Elizabeth	Eastern Cape	34°0'6" S, 25°31'40" E	adult thigh
AC 3239	St. Francis Bay	St. Francis Bay	Eastern Cape	34° 9'36.47"S, 24°49'26.31"E	adult thigh
AC 3240	St. Francis Bay	St. Francis Bay	Eastern Cape	34° 9'36.47"S, 24°49'26.31"E	adult thigh
DNA 0102	Lovemore Heights, PE	Port Elizabeth	Eastern Cape	34°0'4.8" S, 25°31'38.1" E	adult thigh
DNA 0105	Seal Point Nature Reserve	Cape St. Francis	Eastern Cape	34°12'25" S, 24°49'6.9" E	tadpole tail tip
WC 10-104	Seal Point Nature Reserve	Cape St. Francis	Eastern Cape	34°12'25" S, 24°49'6.9" E	tadpole tail tip
WC 10-125	Seal Point Nature Reserve	Cape St. Francis	Eastern Cape	34°12'25" S, 24°49'6.9" E	tadpole tail tip
WC 10-149	Seal Point Nature Reserve	Cape St. Francis	Eastern Cape	34°12'25" S, 24°49'6.9" E	tadpole tail tip
WC 10-150	Seal Point Nature Reserve	Cape St. Francis	Eastern Cape	34°12'25" S, 24°49'6.9" E	tadpole tail tip
AC 3241	Springbok	Namaqualand	Northern Cape	29°41'3.28"S, 17°53'14.45"E	tadpole tail tip
AC 3242	Springbok	Namaqualand	Northern Cape	29°41'3.28"S, 17°53'14.45"E	tadpole tail tip
AC 3243	Springbok	Namaqualand	Northern Cape	29°41'3.28"S, 17°53'14.45"E	tadpole tail tip
AC 3244	Springbok	Namaqualand	Northern Cape	29°41'3.28"S, 17°53'14.45"E	tadpole tail tip

AC 3245	Springbok	Namaqualand	Northern Cape	29°41'3.28"S, 17°53'14.45"E	tadpole tail tip
AC 3246	Buffels River	Namaqualand	Northern Cape	29°41'3.28"S, 17°53'14.45"E	tadpole tail tip
AC 3247	Buffels River	Namaqualand	Northern Cape	29°59'30.71"S, 17°52'35.50"E	tadpole tail tip
AC 3248	Buffels River	Namaqualand	Northern Cape	29°59'30.71"S, 17°52'35.50"E	tadpole tail tip
AC 3249	Buffels River	Namaqualand	Northern Cape	29°59'30.71"S, 17°52'35.50"E	tadpole tail tip
AC 3250	Buffels River	Namaqualand	Northern Cape	29°59'30.71"S, 17°52'35.50"E	tadpole tail tip
AC 3251	Buffels River	Namaqualand	Northern Cape	29°59'30.71"S, 17°52'35.50"E	tadpole tail tip
AC 3252	Buffels River	Namaqualand	Northern Cape	29°59'30.71"S, 17°52'35.50"E	tadpole tail tip
AC 3253	Namaqualand	Namaqualand	Northern Cape	33° 35' 52" S, 26° 54' 07" E	tadpole tail tip
AC 3254	Namaqualand	Namaqualand	Northern Cape	33° 35' 52" S, 26° 54' 07" E	tadpole tail tip
AC 3255	Namaqualand	Namaqualand	Northern Cape	33° 35' 52" S, 26° 54' 07" E	tadpole tail tip
RB0409-005	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip
RB0409-007	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip
RB0409-008	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip
RB0409-009	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip
RB0409-010	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip
RB0409-011	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip
RB0409-014	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip
RB0409-015	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip
RB0409-017	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip
RB0409-019	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip
RB11-E028	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip
RB11-E034	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip
RB11-E035	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip
RB11-E036	East Beach	Port Alfred	Eastern Cape	33° 35' 52" S, 26° 54' 07" E	adult toe clip

Table 2: Other sequences used in the comparative analysis listing species, country, region, latitude, longitude, the GenBank accession number and reference.

Species	Country	Region	Lattitude	Longitude	GenBank	Author
Amietophrynus rangeri	South Africa	Pietermaritzburg	-29.60036	30.37936	AF220868	Cunningham & Cherry, 2000
A. rangeri	South Africa	Pietermaritzburg	-29.60036	30.37936	AF220869	Cunningham & Cherry, 2000
A. rangeri	South Africa	Weza	-30.59202	29.74598	AF220868	Cunningham & Cherry, 2000
A. rangeri	South Africa	Bloefontein	-29.11835	26.22492	AF220871	Cunningham & Cherry, 2000
A. rangeri	South Africa	Grahamstown	-33.30566	26.52453	AF220870	Cunningham & Cherry, 2000
A. rangeri	South Africa	Stellenbosch	-33.92318	18.86531	AF220872	Cunningham & Cherry, 2000
A. rangeri	South Africa	Stellenbosch	-33.92318	18.86531	AF220873	Cunningham & Cherry, 2000
A. rangeri	South Africa	Cederburg	-32.32056	19.10329	AF220874	Cunningham & Cherry, 200
A.regularis	Niger	Tapou	12.47480	2.42760	HM77002	Vasconcelos *et al.* 2010
A.regularis	Niger	Tapou	12.47480	2.42760	HM770003	Vasconcelos *et al.* 2010
A.regularis	Burkina Faso	Gourma	12.06033	0.36933	HM770004	Vasconcelos *et al.* 2010
A.regularis	Burkina Faso	Gourma	12.06033	0.36933	HM770005	Vasconcelos *et al.* 2010
A.regularis	Faso	Gourma	12.06033	0.36933	HM770006	Vasconcelos *et al.* 2010
A.regularis	Mali	Kayes	14.50400	-11.09098	HM770007	Vasconcelos *et al.* 2010
A.regularis	Guinea-Bissau	Bissau	11.86031	-15.57870	HM770008	Vasconcelos*et al.* 2010
A.regularis	Guinea-Bissau	Bissau	11.86031	-15.57870	HM770009	Vasconcelos *et al.* 2010
Phrynobatrachus parvulus	Malawi	Nyika Plateau	-10.58330	33.80000	FJ829306	Zimkus & Schick, 2010
P. parvulus	Tanzania	Rubeho Managalisa Forest Reserve	-7.16670	36.41670	FJ829309	Zimkus & Schick, 2010
P. parvulus	Tanzania	Rubeho Managalisa Forest Reserve	-7.16670	36.41670	FJ829310	Zimkus & Schick, 2010
P. parvulus	Tanzania	West Kilombero Scarp	-7.91670	36.50000	FJ829307	Zimkus & Schick, 2010

		Forest Reserve, Udzungwa Mountains West Kilombero Scarp Forest Reserve,				
P. parvulus	Tanzania	Udzungwa Mountains	-7.91670	36.50000	FJ829308	Zimkus & Schick, 2010
P. parvulus	Uganda	Bundibugyo District, Bundibugyo	0.67680	30.06940	FJ889465	Zimkus & Schick, 2010
P. parvulus	Uganda	Bundibugyo District, Bundibugyo	0.67680	30.06940	FJ889466	Zimkus & Schick, 2010
P. parvulus	Uganda	Bundibugyo District, Bundibugyo	0.67680	30.06940	FJ889467	Zimkus & Schick, 2010
P. parvulus	Uganda	Bundibugyo District, Bundibugyo	0.67680	30.06940	FJ889468	Zimkus & Schick, 2010
P. parvulus	Malawi	Nyika Plateau	-10.58330	33.80000	EU075296	Zimkus
P. parvulus	Malawi	Nyika Plateau	-10.58330	33.80000	EU075297	Zimkus
P. parvulus	Malawi	Nyika Plateau	-10.58330	33.80000	EU075298	Zimkus
P. parvulus	Uganda	Rwenzori Mountains, Bundibugyo	0.55165	29.95036	GQ183589	Siow *et al.,* 2009 (Unpublished)
P. parvulus	Uganda	Rwenzori Mountains, Bundibugyo	0.55165	29.95036	GQ183590	Siow *et al.,* 2009 (Unpublished)
P. parvulus	Uganda	Rwenzori Mountains, Bundibugyo	0.55165	29.95036	GQ183588	Siow *et al.,* 2009 (Unpublished)
Ptycahdena anchietae	Tanzania	Dar Es Salaam University, Dar Es Salaam	-6.74711	39.20536	KC179964	de Sa *et al. 2012*
P. anchietae	Kenya	Karacha Pools, Arabuko Sokoke Forest	-3.39382	39.87995	DQ525920	Measey *et al.*2007
P. anchietae	Somalia	Karin, Bari Region	10.98264	49.21954	DQ525921	Measey *et al.*2007
P. anchietae	Somalia	Karin, Bari Region	10.98264	49.21954	DQ525922	Measey *et al.*2007
P. anchietae	Kenya	Kakamega Forest	0.26667	4447.48	AY517609	Vences *et al.* 2004
P. anchietae	Tanzania	Makuyuni	-4.07986	38.10333	AY517610	Vences *et al.* 2004
P. anchietae	Kenya	Runda-Gigiri	-1.23135	36.80768	AY517611	Vences *et al.* 2004
P. anchietae	Kenya	Runda-Gigiri	-1.23135	36.80768	AY517612	Vences *et al.* 2004

P. anchietae	Uganda	Semliki National Park	0.80554	30.04041	GQ183596	Siow *et al*., 2009 (Unpublished)
P. anchietae	Uganda	Semliki National Park	0.80554	30.04041	GQ183597	Siow *et al*., 2009 (Unpublished)
P. anchietae	Uganda	Semliki National Park	0.80554	30.04041	GQ183598	Siow *et al*., 2009 (Unpublished)

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Appendix

Recipes used for chemical solutions used in the DNA Extraction.

Sodium Chloride-Tris-EDTA buffer (STE):
Sodium Chloride – 0.58 g
Tris – 0.6g
Ethylenediaminetetraacetic acid (EDTA) – 0.037 g
Distilled Water – 100ml

Sodium dodecyl sulfate solution (10 %) (SDS):
Sodium dodecyl sulfate – 10 g
Distilled Water – 100 ml

Extraction Buffer:
STE – 50ml
SDS (10 %) – 7.5ml

Tris-EDTA buffer (TE):
Tris – 0.12 g
EDTA – 0.037g
Distilled Water – 100 ml