A Phylogenetic History of the Tenerife Skink *Chalcides viridanus:* A multi-locus Coalescent Approach

Matthew Woods

A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Masters of Philosophy.

March 2015

Abstract

Previous studies on the Tenerife skink *Chalcides viridanus* found clear links between observed within-island cladogenesis and the geological history of the island. Since these studies there have been many advances in conceptual, numerical and methodological approaches in phylogenetic analyses. This study aims to revisit the phylogeography of the Tenerife skink, using more current *BEAST analysis techniques, and attempt to resolve some unanswered questions about population history of this species. Specifically, using previously unused nuclear gene markers to obtain a more robust phylogeographical history of the species, using new techniques to estimate whether any historic changes in population size can be linked to known geological events and whether there is enough evidence to reclassify any discovered genetic clades as distinct species.

A multi-locus approach was undertaken, using more informative mtDNA gene fragments (Cyt-b & ND1 & 2, totalling 1566bp) as well as the sequencing of 5 nuclear loci (PRLR-555bp, Rag-1-761bp, RELN-583bp, EXPH-796bp and SELT-414bp). These sequences were combined with the latest Bayesian methods to estimate divergence times, historical changes in population structure and infer species boundaries. Results from the BAPS and *BEAST analyses identified three main population groups within the island, the geographical distribution of two of which were restricted to the areas representing the ancient precursor islands Teno & Anaga in the North West and North East of Tenerife, respectively. Contrary to previous findings, the divergence estimates reveal the NE lineage was first to diverge ~1.16 Ma, with the NW diverging ~ 0.6 Ma. The third population group is widely geographically distributed across the island consisting of two clades, one previously unidentified. Results show evidence of a substantial population expansion in this population group approximately 0.2 Ma, which ties in with the end of the last major eruptive cycle. Species delimitation analyses seem to favour a one species model for *C. viridanus*, however the effects of incomplete lineage sorting and low diversity in the nuclear loci make the robustness of this result questionable.

Overall this study highlights both how using a multi-locus coalescent approach can reveal a greater insight in to a species' phylogeographical history, but also how factors such as incomplete lineage sorting can severely limit any intraspecific phylogenetic inferences made on recently diverged population groups.

Contents

List of abbreviations

BAPS - Bayesian Analysis of genetic Population Structure.

PCR - Polymerase chain reaction.

mtDNA – Mitochondrial DNA.

nuDNA – Nuclear DNA.

*BEAST - Bayesian Evolutionary Analysis Sampling Trees.

BSP – Bayesian Skyline Plot.

MCMC – Markov Chain Monte Carlo.

rjMCMC – Reversible Jump Markov Chain Monte Carlo.

TFW – Tenerife western clade.

TFC – Tenerife central clade.

TFE – Tenerife eastern clade.

GCN – Gran Canaria northern clade.

GOM – La Gomera

HER – El Hierro

NW – North West

NE – North East

Ma – Million Years

Mya – Million Years Ago

RELN – Reelin gene Fragment.

EXPH – Exophilin gene Fragment.

RAG1 – Recombination activating gene fragment.

PRLR – Prolactin receptor gene fragment.

Cytb – Cytochrome B gene fragment.

SELT – Selanoprotein T gene fragment.

1. General Introduction

1.1 Phylogeography – A History

The term phylogeography was first introduced almost three decades ago (Avise *et al.* 1987) and it refers to the phylogenetic analysis of molecular data in the context of the geographic distribution of the organism. It is a field that emerged almost of necessity with the aim to unite the fields of phylogenetics and population genetics, building on the long established principles of genetics in the origin and evolution of animal species (Dobzhansky 1937; Mayr 1963). Avise's seminal work in 1987 suggested that genetic data from multiple co-distributed taxa could play an important part in answering deep seated questions about how geographical, climatological or even past geological factors have affected the current observed distribution of biodiversity today (Hickerson *et al*. 2010). Factors that affect the distribution of morphological and genetic variation within and between vertebrate populations are of key interest in the field of evolutionary biology and understanding them is essential for developing accurate models of speciation (Barton & Charlesworth, 1984; Barton & Hewitt, 1989). Phylogeography is highly integrative discipline, which aims to combine molecular genealogical evidence with independent information from fields such as geology, ethology, population genetics, phylogenetic biology and palaeontology to gain a detailed insight of historical population processes and the spatial distributions of morphological traits (Avise, 2000; Gübitz *et al.,* 2005; Juan *et al.*, 2000).

Historical factors can leave deep-seated distinctive patterns of geographical variation within populations (Thorpe, 1975; 1979). Allopatric speciation can occur when a population becomes isolated by geological events, such as volcanism or after the colonisation of an isolated region such as a recently emerged island. Lineages will then diverge due to factors such as genetic drift and potentially by different selection pressures mediated by ecological conditions. Further variation can arise from subsequent changes in the range of the populations or introgression from secondary contacts of populations (Thorpe, 1987).

Numerous studies on a wide range or organisms have demonstrated evidence of historical fragmentation of gene flow over continental areas (Hewitt, 1996; Hewitt, 2004; Taberlet *et al.*

1998). However, population differentiation over much smaller areas can be more easily studied and allow for more decisive findings. For these reasons, oceanic islands provide excellent locations to examine the effects of historical events such as colonisation, isolation of populations due to geological activity, bottlenecks and selective sweeps (Juan *et al.* 1998). It has been observed that species on small oceanic islands can express considerable intraspecific variation, both morphologically and genetically. When combined with a geologically violent past, islands present a clear model to investigate the causes of substantial genetic variability over relatively small distances (Thorpe *et al.* 1995; Juan *et al.* 1996, 1998, 2000; Thorpe & Malhotra. 1996; Pestano & Brown 1999). One of the main reasons why small volcanic oceanic islands are excellent models for examining intraspecific variation is that they generally have many of the variables required to fuel the numerous processes that lead to adaption. They often possess extreme variability in altitude and this, coupled with prevailing trade winds, can lead to highly variable ecological biomes across an island which can have a significant effect on the variation of morphology across relatively short distances (Brown & Thorpe 1991, Brown *et al.* 1991). There are also many geological processes that have been described to explain the presence of geographical variation on small islands including vicariance associated with lava flows (Carson *et al.* 1990, Gübitz *et al.* 2005, Pestano & Brown 1999, Thorpe 2002), the union of formally separate islands containing distinct lineages (Brown *et al.* 2000, Gübitz *et al.* 2000, Thorpe *et al.* 1996) and events such as major landslides (Brown *et al.* 2006) and partial island submergence (Gifford *et al.* 2004, Glor *et al.* 2004).

Prior to the use of PCR-based DNA sequencing, explaining these ecological relationships between geographical variation in a population with what is known about the historical geological events was generally achieved by comparing data on morphological variations with data from geological sources (e.g. Lopez-Jurado & Baez 1985). However not all genetic variation is detectable through morphology alone and in order to collect a more complete picture of a population history, genetic comparisons are essential. Fundamental to the successful development of phylogeography as one of the key tools in understanding modern day biodiversity was the analysis of mitochondrial DNA (mtDNA) at the species level (Hickerson *et al.* 2010).

Due to the increase in computational power as well as the development of molecular markers and new lab techniques over the last three decades, the field of phylogeography has become a key tool in constructing a species biogeographical past.

1.2 The use of Molecular Markers in Phylogeography

The most revolutionary idea behind the emerging field of phylogeography, and the reason for its unparalleled success in the field of population genetics was the use of mitochondrial DNA as operational taxonomic units (OTUs) in phylogenetic analyses (Avise 1987). This in practice meant that clades within a gene genealogy of a species were assumed to reflect the boundaries of populations and it allowed for the exploration of the history of clade defined linages within a species.

The vertebrate mitochondrial genome is a circular molecule about 17Kb in length and contains 37 genes. It has several features that have made it very popular for phylogeographic studies, including its high level of variability, molecular simplicity and almost neutral mode of evolution (Avise 1998, 2009). Its rapid evolutionary rates often lead to the discovery of many haplotypes within a species. Substitution rates vary across the mitochondrial genome, this allows for it to have applications across many different types of phylogenetic study. Faster evolving regions of the mt genome can be used for intraspecific variation and the slower evolving regions can be used for interspecific or intrageneric variation. Areas of the mitochondrial control region have very high rates of evolution and the development of markers from this region have led to many studies determining phylogeographic structuring within a population or species (Amato *et al.* 2008, Brown *et al.* 2000, 2006; Gübitz *et al.* 2000, 2005).

Mitochondrial DNA has proved extremely useful in describing population genetic structure. However, it does have some limitations. For example, it only provides information on maternal gene flow as well as being non-recombining, meaning that it allows inference of a single tree that might have quite a different history to genes found across the nuclear genome.

Recently, several studies have questioned whether mtDNA alone is sufficient to reconstruct the population history of a species and recommend that any mitochondrial derived gene tree should be compared with multiple, unlinked nuclear loci (Ballard and Whitlock 2004; Edwards

et al 2005; Bazin *et al.* 2006; Rubinoff & Holland 2005). It has been shown that mtDNA may not always accurately reflect nuclear genome patterns (Ballard & Whitlock 2004). This is due to the fact that because the mitochondrial genome is so small, it is often not possible to get a well resolved gene tree even with high quality mtDNA data, which calls for evidence from other sources such as nuclear DNA (Degnan 1993; Slade *et al*. 1994). In cases where both mtDNA and nuclear DNA have been used to determine gene trees it has been shown that the use of mtDNA alone could, in severe cases, misrepresent the original phylogeny of the species and lead to inaccurate conclusions about population history (Godinho *et al.* 2008; Renoult *et al.* 2009).

In theory, nuclear DNA (nuDNA) sequences can be used in the same manner as mtDNA, with gene trees being inferred from the represented haplotypes in phylogenetic analyses (Zink & Barrowclough 2008). However basing a phylogenetic analysis on a single nuclear locus may not lead to an accurate reconstruction of a species tree (Wiens *et al* 2010) and multilocus phylogenies from multiple unlinked nuclear genes are required to overcome any misleading signals from individual loci (Wiens 1998, Rokas *et al.* 2003). Misleading signals in nuDNA can arise due to incomplete lineage sorting of ancestral polymorphisms or introgression between populations. Incomplete lineage sorting can be problematic when using nuDNA to construct a gene tree within a recently diverged population and can lead to incongruence with mtDNA trees. This is due to the lower substitution rates in nuDNA, which are generally 10 times lower than mtDNA (Brown 1983) and also because nuDNA has an effective population size four times the size of mtDNA. These factors can lead to cases where lineage sorting is complete for mtDNA (showing mtDNA populations to be reciprocally monophyletic), but incomplete for nuDNA (showing nuDNA populations to be paraphyletic or polyphyletic) in geographically allopatric populations. In cases such as this, it is not possible for nuDNA to "confirm" mtDNA, only show that mtDNA is a leading indicator and nuDNA is a lagging indicator (Zink & Barrowclough 2008). Despite these limitations, comparing mtDNA with nuDNA is now expected in the field in order to gain a more robust estimation of a population's phylogeographical history.

1.3 Species Concepts

The concept of a species is recognised as one of the most fundamental units in biology and its importance has even been compared to that of genes, cells and organisms in terms of classification (Mayr 1982, de Queiroz 2007). But despite this recognition, the issue of upon which definitions of species concepts to use when concerning species delimitation has been long contested between different subgroups of biologists. The main problem seems to be that the species concepts these groups are advocating are all at least somewhat irreconcilable, with as many as 24 different species concepts being suggested (Mayden 1997).

Most biologists now agree that a species is the smallest evolutionary unit, a group of organisms following its own evolutionary pathway. The problem arises when attempts are made to diagnose this criteria for populations of said organisms. The main species concepts used as criteria to delimit a species are the Biological Species Concept, the Morphological Species Concept and the Phylogenetic Species Concept. The Biological Species Concept is the classical species concept, which relies on the reproductive isolation of populations leading to organisms moving along different evolutionary pathways (Wright 1940, Mayr 1943). This concept relies on members of the same species being able to reproduce, producing fertile offspring and possessing shared specific mate recognition or fertilisation systems (Paterson 1985). The Biological Species Concept as a method for delimiting species has long been known to have severe limitations, mostly due to the sheer impracticality of observing many organisms reproducing in the wild (Cronquist 1978). However the issue of reproductive incompatibility has been of central importance to those biologists who study hybrid zones (de Queiroz 2007). The Morphological Species Concept classifies organisms into species based on their morphological characteristics. It has long been long been known that there are morphologically distinct groups were variation between these groups had been shown to be discontinuous (Donoghue 1985). Delimiting species this way becomes trickier when examining more closely related organisms, or those who moved about in space and time, but even then morphologically distinct groups could be shown to exist (Gould 1982). The Morphological Species concept however is central to species delimitation for palaeontologists, who often have little else in the way of evidence with which to classify a species. Since the rise of phylogenetics and coalescent theory, the Phylogenetic Species Concept seems to dominate the field of species delimitation. This concept is based on a species being an

irreducible cluster of organisms, diagnosably distinct from other such clusters, with which there is a parental pattern of ancestry and descent (Cracraft 1989). This species concept has several properties, the ancestor becomes extinct when the lineage splits (Hennig 1966), the group is monophyletic (Donoghue 1985) and all the alleles of a given gene are descended from a common ancestral allele not shared with those of other species, therefore making it diagnosable (Avise & Ball 1990). Essentially, and crucially in studies such as this, it is now possible using Bayesian analysis techniques to examine whether or not groups of organisms share genes by testing the genealogical trees to see if alleles are shared between the groups. The levels of molecular divergence between these groups can then be used as one line of evidence for species delimitation (de Quieroz 2007).

1.4 Subspecies concepts

The concept of a subspecies was developed in order to refine distinctions in taxonomy, speciation and to help explain geographical variation. The advancement in genetic techniques has allowed a move away from morphological based taxonomy to a phylogenetic based one in order to describe geographical variation (Mulcahy, 2008). These techniques can be used in order to sequence mitochondrial or nuclear genes and then analyse the genetic distance between apparent subspecies as well as determine their genetic distinctiveness, or evaluate evolutionary lineages (Burbrink, 2002; Fritz et al., 2007). If subspecies are found to have high genetic distance values, or there is evidence of distinct evolutionary lineages, then these subspecies groups can be delimited in to distinct species (Makowsky et al., 2010)

The validity of subspecies has been brought in to question since the advancement of genetic techniques as the results from phylogenetic analyses are often discordant with the recognised subspecies as categorised by morphological data (Ball and Avise 1992, Phillimore and Owens 2006)

After the genetic analyses of 41 avian species, Zink (2004) found that only 3% of avian subspecies had sufficient grounds for being recognised as distinct evolutionary units, as well as finding the average bird species to have less than half the amount of evolving groups when compared to studies based on morphological and geographical data. The conclusion was that, whilst the number of distinct avian species should remain the same, there were too many subspecies. Similar herpetological studies suggested that genetic sequencing also significantly

altered reptile taxonomy by increasing the number of species and decreasing the number of subspecies (Torstrom *et al.* 2014). It has been argued (maybe somewhat controversially) that the subspecies concept could be an out of date one, and that the concept may not represent evolutionary relationships in most cases, but may merely just reflect the human need to categorise.

1.5 The Canary Islands

The Canary Islands are located between latitudes 27°37' and 29°25'N, 13°20' and 18°10'W, with the most eastern edge situated one hundred kilometres from the northwest African coast. They consist of seven independently formed islands, along with several smaller islets extending 500km across the eastern Atlantic. The seven main volcanic islands are Lanzarote, Fuerteventura, Gran Canaria, Tenerife, La Palma, La Gomera and El Hierro (**Figure1**).

Figure 1 Map of the Canary Islands

The islands formed through a series of volcanic events through the Miocene (~20 Mya) to the late Pleistocene (0.5 Mya) (Staudigel *et al.* 1986) with the geological history of the islands being well documented (Ancochea 1990, Cantagrel *et al*. 1984). The islands have a known sequence of sub aerial appearances and therefore the timings they became available for colonisation from the surrounding areas is well understood (Juan *et al.*, 2000). The first appearances of the islands are thought to be attributed to the movements of the African and European plates in the upper cretaceous (Le Bas *et al.* 1986).

The islands emerged in a general western progression with the westerly Fuerteventura (20.6 Mya) and Lanzarote (15.5 Mya) emerging first followed by Gran Canaria (14.5 Mya), Tenerife (11.6 Mya), La Gomera (12 Mya) and the two most easterly islands La Palma (1.77 Mya) and El Hierro (1.12 Mya) emerging much later (Ancochea 1990, Cantagrel *et al*. 1984 Guillou *et al.* 1996). Each island formed through independent volcanic events and none of the current islands have ever been joined above sea level meaning that any between island population patterns are due to colonisation events (Juan *et al*. 2000).

While the time of the first emergence of all the islands is well known, it is important to recognise for phylogeographical reasons that the islands have been continuously volcanically active since emergence. All but one (La Gomera) have been active in the last million years, with several (Lanzarote, Tenerife, La Palma and El Hierro) having experienced eruptions within the last 1000 years (Ancochea 1990, Le Bas *et al.* 1986, Cantagrel *et al*. 1984, Guillou *et al.* 1996.)

The Canarian flora and fauna are varied and numerous, with approximately 1000 species of plants and over 6000 species of invertebrates. The native species show high levels of endemism with about 27% of plants as well as 50% of invertebrates being endemic to the archipelago (Juan *et al.* 2000). There are currently 13 extent reptile species present on the islands, with 12 of those also being endemic to the islands (Pleguezuelos *et al*. 2002) as well as several species of bat, three rodents (now extinct) and two species of shrew. Due to the prevailing winds and sea currents, the two most probable sources for colonisers are neighbouring North Africa and the Iberian Peninsula. Many phylogenetic studies have since confirmed this theory, presenting a stepping stone model of island colonisation from North Africa and across the Canary islands with a stepwise dispersal from the oldest islands to the youngest. The *Gallotia galloti* lizard, for example, appears to have dispersed from Tenerife along two independent pathways, one from north Tenerife to La Palma and one from south Tenerife to La Gomera and El Hierro (Thorpe 1994; Thorpe & Malhotra 1998; Gonzalez 1996). The darkling beetle species *Pimelia and Hegeter* (Juan 1995; 1996) are also compatible with the stepping stone model, as well as *Drosophila subobscura* (Pinto *et al.* 1997) the *Gonepteryx* brimstone butterflies (Brunton & Hurst 1998) *Dysdera* spiders (Arnedo 1996) & *Steganacarid* mites (Avanzati *et al.* 1994). There is also evidence of some back colonisation in several species (Juan *et al.* 2000).

The humid trade winds from the northeast, in combination with the altitude of the volcanoes means each of the islands contain many variable vegetational zones (**Figure 2**). This combined with the multitude of vacariance events produced by each islands volcanic events mean island intraspecific biodiversity is high. A study on island-wide geographical variation in the Gran Canarian skink *Chalcides sexlineatus* using fragments of the mitochondrial 12S ribosomal RNA gene discovered three divergent lineages associated with the Northern, South Eastern and South Western parts of the island (Pestano & Brown 1999). This phylogeographic structuring was consistent with known historical volcanic activity on the island. Other Gran Canaria species including the gecko *Tarentola boettgeri* (Nogales *et al.* 1998, Gübitz *et al.* 2005) and the darkling beetle *Pimela sparsa* (Contreras-Diaz *et al.* 2003) also have similar phylogeographies which is consistent with simple models of fragmentation relating to the volcanic activity. Tenerife on the other hand, has a much more complex geological history.

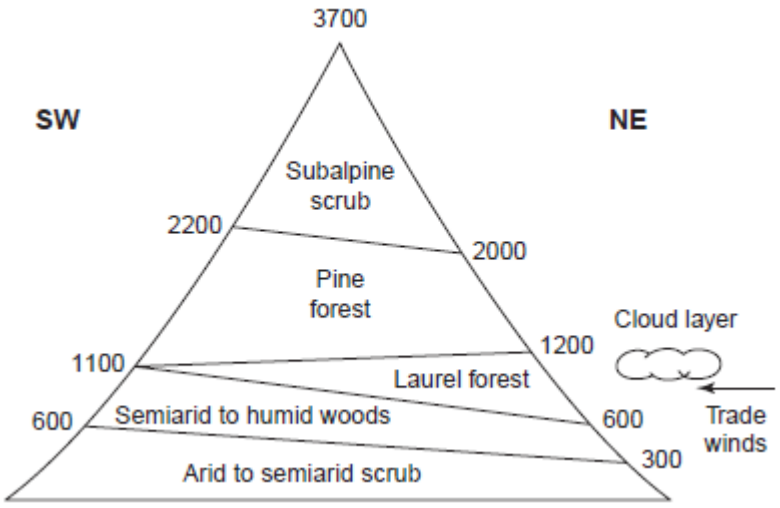


Figure 2 - An example of the general pattern of the vegetational zones on the Canary Islands (Juan et al 2002).

The Canary Islands are a great model region for studies of intraspecific variation, with many phylogeographical studies of the lizard, insect and other native species having been carried out.

1.4 Tenerife

The island of Tenerife is the largest (2058km^2) and highest (3718m) of the Canaries and possesses one of the most complex geological histories. Dating has revealed three areas of the island that are older than the rest. These three deeply eroded edifices consist of Anaga in the NE, Teno in the NW and Adeje in the southern part of the island (**Figure 3**). These so called precursor islands are thought to be 6.5, 7.5 and 11.6 million years old respectively (Guillou 2004). Tenerife has been has been the site of many violent volcanic events throughout its entire history. These volcanic cycles originally formed the main island, with the central Cañadas edifice uniting Teno, Anaga and Adeje between 3.5 and 2.7 Mya and further eruption cycles occurring between 2.5 - 1.4 Mya and 1.1 - 0.2Mya (Ancochea, 1999). There is no evidence to suggest these precursor islands were ever joined prior to this, which is important when considering explanations of within-island patterns of genetic variation.

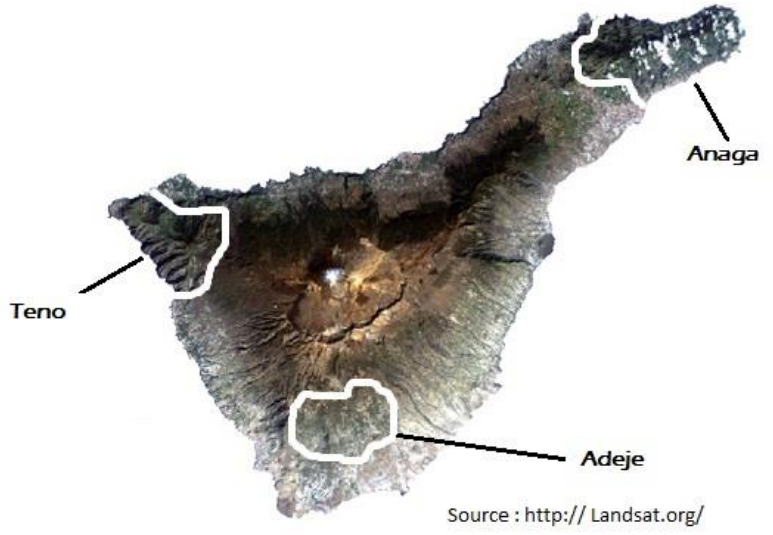


Figure 3 Tenerife with approximate regions of precursor islands highlighted.

There is now strong evidence that different aspects of Tenerife's volcanic history are intimately linked with the phylogeography and genetic variation of some of the islands reptile species. For example, a repeated pattern of three clades associated with the three precursor islands is observed in many diverse organisms on Tenerife. *Dysdera* spiders, *Pimelia,*

Eutrichopus & *Calathus* beetles, *Gallotia* lizards, *Steganecarus* mites and *Loboptera* cockroaches all show similar patterns of cladogenisis on the island, though their levels of divergence varies considerable between the species (Ancochea *et al.* 1990; Arnedo 1996; Contreras-Diaz *et al.* 2003; Pinto *et al.* 1997). There is also evidence of debris avalanches causing fragmentation and isolation of *Gallotia galloti* are thought to have caused the observed within island cladogenesis of this species (Brown *et al*. 2006). There is also evidence that similar geological events have led to secondary contact and recent bottle necks /expansion, which have shaped the genetic variation of *Tarentola delalandii* (Gübitz, 2000).

1.5 Model species: *Chalcides viridanus*

The *Chalcides* genus are a group of lizards belonging to the Scincidae family, better known as skinks. There are currently thought to be around 24 species in this group, with the majority of these occurring in Morocco and the surrounding areas of North Africa, although their spatial distribution spreads north up to areas of southern Europe, as far east as Somalia and Kenya and even across Arabia as far as Pakistan (Carranza *et al.* 2008). Most species of *Chalcides* spend much time in the topmost layers of lose soil and litter or sand, depending on their habitats. Many of these species have relatively elongated bodies, most likely evolved to cope with the locomotory problems of moving in such environments (Caputo *et al.* 1995). Many of the more primitive *Chalcides* skinks spend most of their time hidden and are generally more active at night, but many members of the genus appear to be partly heliothermic and have been observed basking in the sun at times. All *Chalcides* skinks possess a transparent window in their lower eyelids, this allows them to bask with their eyes closed whilst still retaining some vision, a trait that allows to both be more aware of predators and also reduce moisture loss whilst basking (Arnold 1973, Greer 1983).

A single species of *Chalcides* resides on all but one of the seven Canary Islands. These consist of four endemic species that show considerable within and between island variation. *Chalcides sexlineatus* is endemic to the central island of Gran Canaria, *C. viridanus* is endemic to the island of Tenerife, *C. coeruleopunctatus* is endemic to the western islands of El Hierro and La Gomera and was originally thought to be part of the *C. viridanus* species (Brown & Pestano 1998, Juan *et al.* 2000) and *C. simonyi* that is endemic to the island of Fuerteventura.

With regards to their original colonisation of the Canary Islands, it is thought that there have probably been two independent transmarine colonisations by a lineage from western Morocco, with the first ancestor of *C. sexlineatus* and *C. viridanus* reaching the central and western islands up to 7 million years ago and rapidly spreading to Gran Canaria, Tenerife and La Gomera between 5-7 million years ago (Juan *et al.* 2000, Carranza *et al*. 2008). Evidence suggests that the spread to the youngest of the Canary islands, El Hierro happened much later, after it rose from the sea approximately 1 million years ago (Guillou *et al.* 1996). Strangely, there is no evidence to suggest *Chalcides* have ever colonised the second youngest of the islands La palma.

For the purposes of this study I will be focusing on *Chalcides viridanus* (**Figure 4**). As previously mentioned it is endemic to Tenerife and is distributed over much of the island up to altitudes of 2000m, apart from a significant strip along the north-east coast.

Figure 4 A captured Chalcides viridanus individual.

There is considerable morphological variation within the island, with a north/south change in body dimensions and scalation (Brown *et al*. 1993). The north of the island provides a lush habitat due to the north/north-east trade winds providing cloud formation on the north facing slopes of Teide. This leads to an increase in rain fall and vegetation along the northern coast. The southern region of the island is considerably more arid. Cloud formation is sparse in the south which leads to increased temperatures and lower rainfall. Because of this the vegetation in the south consists almost exclusively of xerophytic plants, providing little vegetation cover from predators (Fernandopulle 1976). *Chalcides viridanus* individuals in the arid southern habitat possess a blue-green dorsal-tail coloration through to adulthood whereas individuals in the northern lush habitat lose this coloration as they reach adulthood

and instead have dark, uniform dorsal-tail coloration. The tail autotomy of *C. viridanus* makes this variation likely to be due to differing anti-predator strategies between the two environments. A significant decrease in vegetation coverage as well as increased Shrike (*Lanius excubitor*) numbers in the south of the island would seem like logical reasons to explain why a strategy in which predators are attracted to the lizard's tail is favoured (Brown *et al.* 1991).

Molecular studies on *C. viridanus* have since shown that the geographical variation mentioned above is discordant with the phylogeographical patterns over the island. Within island divergence of this species is thought to be linked to historical volcanic activity during the formation of Tenerife. Colonisation is most likely to have occurred ~4 Mya (Brown & Pestano 2000). Analysis of mtDNA provides evidence of two distinct clades in the northwest (Teno) and northeast (Anaga) of the island and a third clade comprising of the majority of the individuals in the central region. The large central clade is thought to have undergone a major expansion around 300,000 years ago, which corresponds with the end of the last major eruptive cycle (Brown *et al.* 2000). The expectation would be that since colonisation occurred only ~0.5 Ma before the volcanic events that began to join the precursor islands, the cladogenisis resulting in the three divergent lineages would have occurred not long afterwards. However this is not supported by the mtDNA tree, which dates the divergence of the central and north-eastern lineages at ~0.9 Mya and the divergence of the north-western ~1.1 Mya. There is currently no evidence to suggest whether this was due to vicariance or colonisation events (Brown *et al.* 2000) so the cause for this discordance is unknown.

So far, phylogeographical inferences about the lizard species of Tenerife have been derived from short and relatively slowly evolving mitochondrial DNA (mtDNA) sequences. The purpose of this project is to sequence more informative mtDNA genes along with several nuclear genes from samples of *Chalcides viridanus* to obtain a more complete picture of the genetic diversity and phylogeographic history within the species *C. viridanus* using a multilocus coalescent approach. This will allow a re-examination of the hypothesis that within island evolution of *C. viridanus* is associated with the geological history of the island (Brown *et al*. 2000). To date, no study has examined whether any of the divergent populations on the Canary Islands represent distinct species, but new statistical techniques will allow for assessment of whether divergent lineages represent distinct species within the island.

2. Materials and Methods

2.1 Sample Collection

Tissue samples, previously collected by Professor Richard Brown, were available for use in this study. The samples had been collected from 36 locations covering most areas of the known distribution of *Chalcides viridanus* on Tenerife. Two individuals were sampled from each site where available. Sampling low numbers of individuals from a large number of sites has been found to be a more effective sampling method than obtaining large samples sizes from fewer sites, because low levels of introgression leading to decreased within site genetic diversity. This increased the chances of all major lineages being found during phylogenetic analyses (Thorpe *et al.* 1996, Brown & Pestano 1998, Brown *et al.* 2000).

Adults were captured using a trapping method involving plastic bottles and tomatoes as bait. Tail tips samples were carefully excised from the captured specimens, after which they were subsequently released back into the wild. The tail tips were immediately stored in 99% alcohol for tissue preservation, and placed in storage at -20°C once they had been returned to the lab. A total of 64 individuals of the species *Chalcides viridanus* were available for DNA extraction.

Along with *C. viridanus* samples I sequenced nuclear genes from 4 samples of *Chalcides coeruleopunctatus* from the nearby islands La Gomera and El Hierro and 4 samples of *Chalcides sexlineatus* from Gran Canaria for use as an out-group in phylogenetic analyses to provide time calibrations on the phylogeny.

In total 72 individuals encompassing 2 species were used in this study (shown in **Table 1**).

Table 1. Specimen details

Specimen	Site	Latitude	Longitude
1.1	Palmmar	N 28 01.409'	W 16 41.374'
1.2	Palmmar	N 28 01.387'	W 16 41.398'
2.1	Guaza	N 28 02.397'	W 16 40.625'
2.2	Guaza	N 28 02.422'	W 16 41.620'
3.1	Ctra Pto Guimar	N 28 17.968'	W 16 22.810'

3.2	Ctra Pto Guimar	N 28 17.968'	W 16 22.810'
4.1	Igueste	N 28 31.471'	W 16 09.271'
4.2	Igueste	N 28 31.449'	W 16 09.324'
4.3	Igueste	N 28 31.479'	W 16 09.319'
5.1	San Andres	N 28 30.437'	W 16 11.725'
5.2	San Andres	N 28 30.437'	W 16 11.725'
6.1	El Tablero	N 28 24.986'	W 016 19.615'
6.2	El Tablero	N 28 24.999'	W 016 19.628'
7.1	Las Lagunetas	N 28 24.720'	W 016 24.420'
8.1	Las Bodegas	N 28 33.597'	W 16 09.382'
8.2	Las Bodegas	N 28 33.659'	W 16 09.258'
9.1	Taganana	N 28 33.641'	W 16 12.849'
9.2	Taganana	N 28 33.580'	W 16 12.991'
9.3	Taganana	N 28 33.581'	W 16 12.993'
10.1	MonteMercedes/La Laguna rd	N 28 31.215'	W 16 17.613'
11.1	nr Tejina	N 28 32.267'	W 16 21.420'
12.1	Bajamar site	N 28 32.505'	W 16 21.237'
13.1	nr Tacoronte/El pris	N 28 29.898'	W 16 25.205'
13.2	nr Tacoronte/El pris	N 28 29.865'	W 16 25.142'
14.1	Valle Jimenez	N 28 29.478'	W 16 16.432'
14.2	Valle Jimenez	N 28 29.483'	W 16 16.439'
15.1	Igueste-Las caletillas	N 28 22.923'	W 16 22.615'
15.2	Igueste-Las caletillas	N 28 22.924'	W 16 22.612'
16.1	La victoria-Acentejo area	N 28 26.734'	W 16 28.008'
16.2	La victoria-Acentejo area	N 28 26.746'	W 16 28.036'
17.1	nr Pto de la Cruz	N 28 24.035'	W 16 31.284'
17.2	nr Pto de la Cruz	N 28 23.979'	W 16 31.285'
18.1	Realejo Bajo	N 28 23.704'	W 16 35.823'
18.2	Realejo Bajo	N 28 23.714'	W 16 35.773'
19.1	W-San Juan de la Rambla	N 28 23.623'	W 16 39.365'
19.2	W-San Juan de la Rambla	N 28 23.615'	W 16 39.365'

20.1	W-Buenavista	N 28 21.981'	W 16 51.360'
20.2	W-Buenavista	N 28 21.984'	W 16 51.368'
21.1	E-Buenavista	N 28 21.769'	W 16 50.554'
22.1	Los Silos	N 28 22.157'	W 16 49.414'
22.2	Los Silos	N 28 22.163'	W 16 49.420'
23.1	Garachico	N 28 22.226'	W 16 46.804'
23.2	Garachico	N 28 22.240'	W 16 46.808'
24.1	San Marcos	N 28 22.309'	W 16 43.131'
24.2	San Marcos	N 28 22.292'	W 16 43.096'
25.1	Adeje	N 28 05.950'	W 16 44.098
25.2	Adeje	N 28 05.985'	W 16 44.148
26.1	Arminene	N 28 07.728'	W 16 45.674
26.2	Arminene	N 28 07.715'	W 16 45.691
27.1	Playa San Juan	N 28 10.235'	W 16 47.906
28.1	Alcala	N 28 11.965'	W 16 49.428
29.1	Nr playa santiago	N 28 13.011'	W 16 49.998
29.2	Nr playa santiago	N 28 13.042'	W 16 50.017
30.1	Santiago del Teide	N 28 17.925'	W 16 48.900
30.2	Santiago del Teide	N 28 17.962'	W 16 48.932
31.1	Carrizales	N 28 18.970'	W 16 51.319
31.2	Carrizales	N 28 19.050'	W 16 51.404
32.1	El Palmar	N 28 20.061'	W 16 51.107
32.2	El Palmar	N 28 20.178'	W 16 51.094
33.1	Arafo	N 28 19.778'	W 16 24.901
34.1	Grenadilla	N 28 07.009'	W 16 34.894
34.2	Grenadilla	N 28 07.027'	W 16 34.879
35.1	La Grenadilla	N 28 04.331'	W 16 39.241
35.2	La Grenadilla	N 28 04.059'	W 16 39.375
36.1	S Guimar valley	N 28 17.702'	W 16 25.054
G2	Hermigua,La Gomera *(C. coeruleopuntatus)*	N 28 16.720’	W 17 19.220
G3	Calera, La Gomera *(C. coeruleopuntatus)*	N 28 14.430’	W 17 15.507

H1	Valverde, El Hierro *(C. coeruleopuntatus)*	N 27 75.005’	W 18 00.056
H2.3	Frontera, El Hierro *(C. coeruleopuntatus)*	N 27 55.458’	W 18 01.519
TAF 1	Tafira, Gran Canaria (*C. sexlineatus)*	N 28 43.289’	W 15 26.389
TAF 3	Tafira, Gran Canaria (*C. sexlineatus)*	N 28 43.289’	W 15 26.389
T1	Tauro, Gran Canaria (*C. sexlineatus)*	N 27 48.002’	W 15 43.045
T2	Tauro, Gran Canaria (*C. sexlineatus)*	N 27 48.002’	W 15 43.045

2.2 DNA Extraction

Genomic DNA was extracted from the available samples using spin column extraction (NBS Biologicals Spin column Genomic DNA Miniprep kit) following the manufacturers protocols. Extracted samples were immediately stored at 20°C to minimise degradation.

2.3 DNA Amplification and Sequencing

Previous amplification and sequencing of three mtDNA genes (Cytochrome B, NADH1 & NADH2) provided a total of 1566bp of sequence for all samples. I used the polymerase chain reaction (PCR) to attempt to amplify a total of 14 selected nuclear DNA loci. All but 5 of these loci contained no variable sites and were therefore non-informative for phylogenetic analysis. The 5 partial genes that did contain small numbers of SNPs were PRLR (555bp), Rag-1 (761bp), RELN (583bp), EXPH (796bp) and SELT (414bp). The primers used I for the amplification of the genes is shown in **Table 2.**

Table 2. Information on the nuclear loci that were tested.

Gene	Primer Name	Primer Sequence (5'-3')	Annealing temperture	Successful Amplification	Variable sites	Source
EXPH5	EXPH5_F1	AATAAACTKGCAGCTATGTACAAAACAAGTC	52	Yes	Yes	Portik *et al.* 2010
	EXPH5_R1	AAYCGCCCTTCTGTGAGTGACCTCT				
RELN	RELN61F	GAGTMACTGAAATAAACTGGGAAAC	53	Yes	Yes	Pinho *et al.* 2009
	RELN62R	GCCATGTAATYCCATTATTTACACTG				
MC1R	MC1RF	GGCNGCCATYGTCAAGAACCGGAACC	51	Yes	No	Pinho *et al.* 2009
	MC1RR	CTCCGRAAGGCRTAAATGATGGGGTCCAC				
PRLR	PRLR-F15	GACARYGARGACCAGCAACTRATGCC	58	Yes	Yes	Townsend *et al.* 2008
	PRLR-R35	GACYTTGTGRACTTCYACRTAATCCAT				
SELT	SELT-F6	GTTATYAGCCAGCGGTACCAAGACATCCG	61	Yes	Yes	Portik *et al.* 2010
	SELT-R6	GCCTATTAAYACTAGTTTGAAGACTGACAG				
P2-07	CCAG630F	CTATGCCCCAATTTCCTTGAT	53	No	N/A	Jackson *et al.* 2011
	CCAG631R	GCCATTGTTCTATCCCACATTT				
P2-47	CCAG636F	AAGATGGCATTTTAGGGAAGGT	53	No	N/A	Jackson *et al.* 2011
	CCAG637R	CATCGCAACAATTTCAAGGTTA				
Rhodopsin	Rhodops_F	GTCCAGCCATCTACCAATCC	49	Yes	No	Hagen *et al.* 2012
	Rhodops_R	CATGATCATTACAGTTACGG				
Unknown intron 4	Unk_intron4_F	TGGACAACATCAAGCCCAC	54	Yes	No	Hagen *et al.* 2012
	Unk_intron4_R	GGTGAACTCCTTGCCAAAG				
KIF24	KIF24_F	SAAACGTRTCTCCMAAACGCATCC	56	Yes	No	Portik *et al.* 2010
	KIF24_R	WGGCTGCTGRAAYTGCTGGTG				
PKDREJ	PKDREJJ1900_F	GTAGTTTCAVCAGGGTGCAAAGGGTATCTTGT	60	Yes	No	Portik *et al.* 2010
	PKDREJ2480_R	TTTCAGTATCTTTDGCCCTTATTTGCCTCATTC				
UBN1	UBN1_F1	CCYCTMAATTTYCTGGCWGARCAGGC	53	No	N/A	Townsend *et al.* 2008
	UBN1_R1	GGTCAGYAAYTTKGCCACHCCYT				
RAG-1	Rag-1_F	TGCACTGTGACATTGGCAA	51	Yes	Yes	Townsend *et al.* 2004
	Rag-1_R	GCCATTCATTTTYCGAA				
RAG-2	Rag-2_F	AACAATGANCTTTCTGATAA	53	No	N/A	Townsend *et al.* 2004
	Rag-2_R	CCTRADGCCAGATATGGYCATA				

I performed PCR using Bioline Biomix Red master mix with the thermo cycling conditions as follows: Initial denaturation at 95°C for 5 minutes followed by 35 cycles of: denaturation at 95°C for 1 minute; annealing at a specific temperature for each fragment (see **Table 2**) for 1 minute and extension at 72°C for 1 minute. The reaction was completed by a final extension at 72°C for 10 minutes. All amplifications were run with negative controls (dH_2O) to check for any possibility of contamination.

Following amplification I subject the PCR products to electrophoresis on 1% agarose gels pre-stained with ethidium bromide and placed under UV-light to ensure successful amplification and check that only a single DNA fragment had been amplified.

Successfully amplified PCR products were purified using the Sigma GenElute PCR Clean-Up Kit and following standard protocol. The purified products were sent away for Sanger sequencing to LGC Genomics (Sanger *et al.* 1977).

2.4 Phylogenetic Analysis

2.5 Sequence Alignment

Once the samples had been successfully sequenced, I checked the sequence chromatograms by eye using the program Bioedit (version 7.0.9.0, Hall 1999). The appearance of "double peaks" on these chromatograms could indicate the presence of heterozygous sites or additional paralogous copies of the gene from elsewhere in the genome. The presence of these paralogous genes or nuclear mitochondrial DNA (Numts) can affect the accuracy of phylogenetic analyses due to the difference in evolutionary rates in comparison to their genuine counterparts (Podnar *et al* 2007, Zhang and Hewitt 1996). It has been shown that paralogous genes often do not accurately reflect the true history of a species and that the ideal molecular marker should be "single copy" (Cruickshank, 2002). The concerns over paralogy have been one of the major reasons for the popularity of organelle genes (such as mitochondrial) in phylogenetic studies, due to their single copy properties. Unfortunately, there are relatively few concrete suggestions for dealing with the problem of paralogous genes except to recognise unusual molecular architecture (Cotton, 2005). Therefore if a sequence, when examined by eye, showed an unusually high number of heterozygous sites I assumed that it was likely to be a paralogous gene and therefore non heterozygous, making any phylogeny inferred from the sequence less reliable. In these cases the locus would not therefore be used.

The mitochondrial genes were combined to form a total of 1566bp of sequence and along with the nuclear genes were aligned using Clustal W (Thompson *et al.* 1994) in the program MEGA (version 5.2, Tamura *et al.* 2011).

2.6 Phylogenetic Construction

I used the program jModelTest2 (Darriba *et al.,* 2012; Guindon & Gascuel, 2003) to determine the best DNA substitution models for further phylogenetic analysis. The program examines up to 56 models in order to determine the best one for the sequence data for each locus. If the best model was unavailable for a particular analysis program then the next most complex was used instead. Each partition was assigned an individual evolutionary model based on the results from jModelTest2.

Prior to the construction of any phylogenetic trees, the program BAPS (Bayesian Analysis of Population Structure) was used to infer population structure from the sequence data (Corander *et al.* 2003). A genetic mixture analysis was carried out to determine the genetic structure within the data set, identifying any genetic clusters.

*BEAST, v 1.7.5 (Drummond *et al.,* 2012) was then used to simultaneously determine phylogenetic relationships and estimate divergence times between the different population groups of *C. viridanus* across Tenerife. *BEAST uses a multispecies coalescent approach but in this case I used population groups as species units. The different haplotypes were assigned to population groups belonging to the Northwest (NW), Northeast (NE) or Central (C) parts of the islands as corresponding with the ancient precursor islands of Tenerife. This was justified using the genetic clusters identified from the BAPS analysis. *Chalcides coeruleopunctatus* from the neighbouring islands El Hierro and La Gomera were diagnosed as being separate species and used as an outgroup for time calibration purposes. The mitochondrial sequence was divided into five functional sets: Cytb codon positions 1 & 2, Cytb codon position 3, NADH codon positions 1 & 2, NADH codon position 3 and tRNA. Sequence diversity was low in the nuclear loci and so they were not partitioned. Appropriate partitioning based on the functional aspects of the sequence like codon position has been shown to be preferable to partitioning by mt gene in order to account for the different substitution processes in multiple genes (Yang & Yoder, 2003; Brandley *et al.*, 2005). Using evidence from previous findings monophyly constraints were applied to the population groups in the following way, (1) the NW, NE and Central Tenerife groups and (2) the El Hierro and La Gomera groups (Brown & Pestano 1998, Brown *et al.* 2000). A Yule prior was used. The prior for the divergence time on the node for the out-group individuals from La Gomera and El Hierro was set hard minimum and maximum limits of 0 and 1.12 to reflect the prior knowledge that El Hierro was colonized from La Gomera around the time of its emergence 1.12mya (Brown & Pestano 1998). All population groups contained more than one individual (at least 7 in this case) which is preferable for this type of analysis (Camargo *et al*. 2012).

Previous evidence suggests that the divergence times for *C. viridanus* on Tenerife are quite recent (Brown *et al.* 2000) and the lack of sequence divergence in shallow trees can lead to some priors being especially influential under a relaxed clock (Brown & Yang 2011). For this reason the data set was run with both a relaxed and then a strict clock and the results

compared. The MCMC chain in *BEAST was run for 20 million generations, with a sampling frequency of 1000, to ensure independence, and a burn-in period of 1 million generations to increase the likelihood that the sampling chain had reached stationarity. Tracer v1.6 was used to examine the posteriors and split standard deviations to ensure stationarity had been reached in the traces as well as to ensure a reasonable effective sample size had been obtained. TreeAnnotator v1.7.5 was used to construct a maximum clade credibility tree from the data.

With the information gained from the *BEAST analysis it was possible to construct estimations of historical demographic changes in the three population groups using Bayesian skyline plots (BSPs) under the piecewise-constant model (Drummound *et al*. 2005). This method uses prior knowledge of substitution rates to estimate changes in effective population size over time. The mtDNA data were partitioned in the same manner as in the *BEAST analysis and a normal prior was specified using the previously estimated substitution rates and their variances from the dating analyses. The BSP approach requires number of groups of coalescent intervals to be specified and this cannot be known *a priori*. The analysis was run a number of times on each of the three population groups (Central clades, northwest clade & northeast clade) with both small (4) and larger (10) numbers of groups and results were similar when compared.

2.7 Species Delimitation

Accurate species delimitation is important in many areas of biology and in this case could have a significant impact on the conservational approach to *C. viridanus* on Tenerife. To date, there has been no attempt to examine whether divergent populations found within the islands in the Canaries could represent distinct species. For the first time, using the program BPP 2.0 (Yang & Rannala 2010, Yang & Rannala 2013), the clades indicated in the genetic data can be examined and the likelihood that they are different species tested. This has been made possible now because multiple loci have been sequenced here for *C. viridanus*. Yang & Rannala proposed BPP as an analysis tool for delimiting species, but openly said they would not discuss species concepts. Nevertheless, the program they have created is based on a lineage examination of shared / unshared alleles between the putative species (Yang & Rannala 2010). Therefore the species concept it is effectively working with is a coalescent, genealogical one (i.e. the Phylogenetic Species Concept). The program BPP 2.0 was used to delimit genetic clusters found using BAPS and examine clades identified by our *BEAST species trees, in order

to determine whether there is enough molecular evidence to warrant *C. viridanus* being reclassified as more than one distinct species. Using user-specified guide trees BPP estimates the probabilities of splits between terminal taxa, assuming no admixture following speciation. I applied BPP analyses to data sets containing both mtDNA and nuDNA, as well as just the nuDNA alone, to examine any dependence on markers and determine the robustness of the results. Individual runs using the reversible jump Markov chain Monte Carlo (rjMCMC) algorithm evaluated subtrees created through the collapsing of nodes present on the guide tree, without branch swapping. All analyses were run for 2,500,000 generations, sampling every 50 generations with a burn-in of 80,000. Population size parameters (Θ) were specified using a gamma prior and runs were performed with priors specifying both a relatively large ancestral population G(1,10) and a relatively small one G(2,2000) and posteriors compared. The age of the root in the species tree (τ) was also assigned from a gamma prior with different runs reflecting both relatively recent divergence times G(2,200) which corresponds to what is already known about *C. viridanus* (Brown *et al.* 2000), whilst other divergence time parameters were assigned from a Dirichlet prior (Yang & Rannala 2010). The differing priors between runs unsurprisingly led to different posterior estimates of both Θ and τ, however the posterior probability of species model estimation was unaffected for each data set. Substitution rates were set accordingly for both mtDNA and nuDNA loci and automatic fine tuning parameters were selected. One of the advantages of the newer BPP version 2.0 is that it offers improved mixing of the rjMCMC which allows for easier switching between species delimitation models compared to the previous version, which was found to poor mixing properties for large or even medium data sets (Yang & Rannala 2013).

3. Results

3.1 Sequence Summary Statistics

The variability of the sequences in terms of percentage of variable sites (**Table 3**) was particularly low for all of the nuclear loci with a mean of only 1.29% variable sites across the 5 nuclear loci. As expected, the proportion of variable sites across the two mtDNA fragments was around 10 times higher than this.

Table 3. Sequence variability of the selected loci expressed as the percentage of variable sites.

Sequence Variability	
Gene	**%**
EXPH	0.50
PRLR	1.48
RAG1	0.92
RELN	1.37
SELT	2.17
mtDNA	15.01

The nucleotide base composition can be seen in **Table 4**.

Table 4. Nucleotide base composition of the selected loci.

Base Composition (%)				
	T(U)	C	A	G
EXPH	24.8	20.4	35.9	19.0
PRLR	22.1	21.9	33.8	22.2
RAG-1	22.5	21.7	33.4	22.4
RELN	20.5	31.5	21.0	27.1
SELT	35.8	13.7	33.7	16.8
mtDNA	26.5	28.1	30.3	15.1

3.2 BAPS Analysis & Networks

The BAPS analysis of all markers identified 4 genetic clusters within the Tenerife population (**Figure 5**). The yellow cluster consists of individuals from only the far North West of Tenerife and the blue cluster of individuals from only the far North East. The green and red clusters contained individuals from sites spanning the rest of the island.

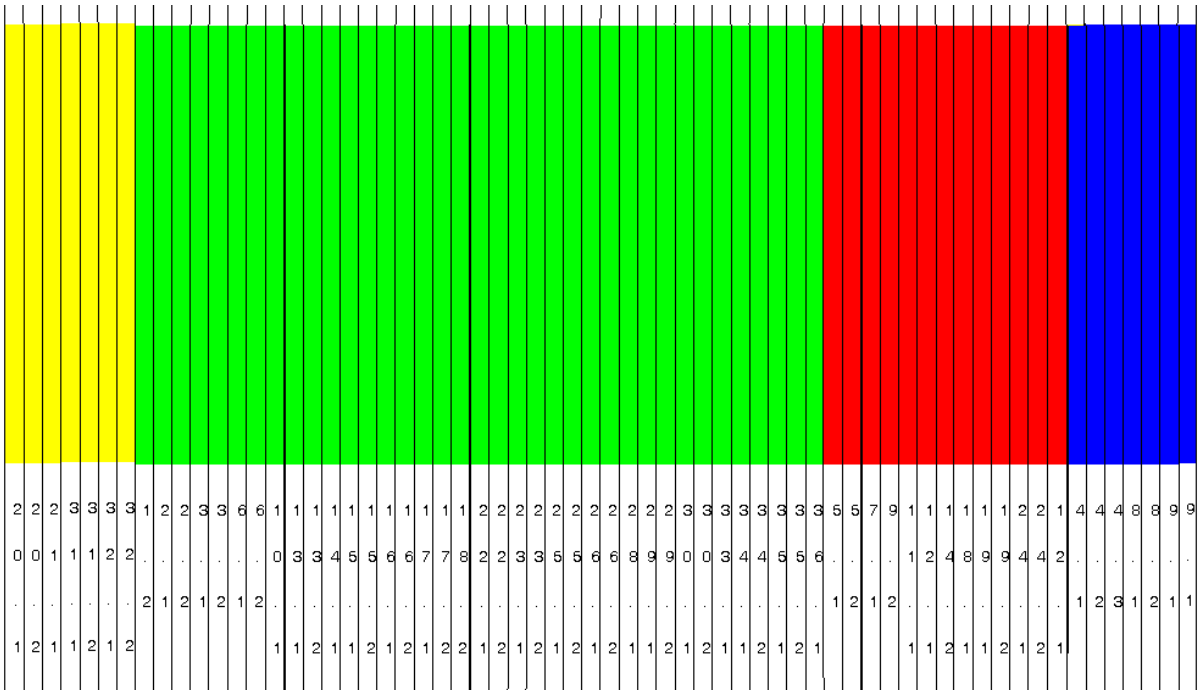
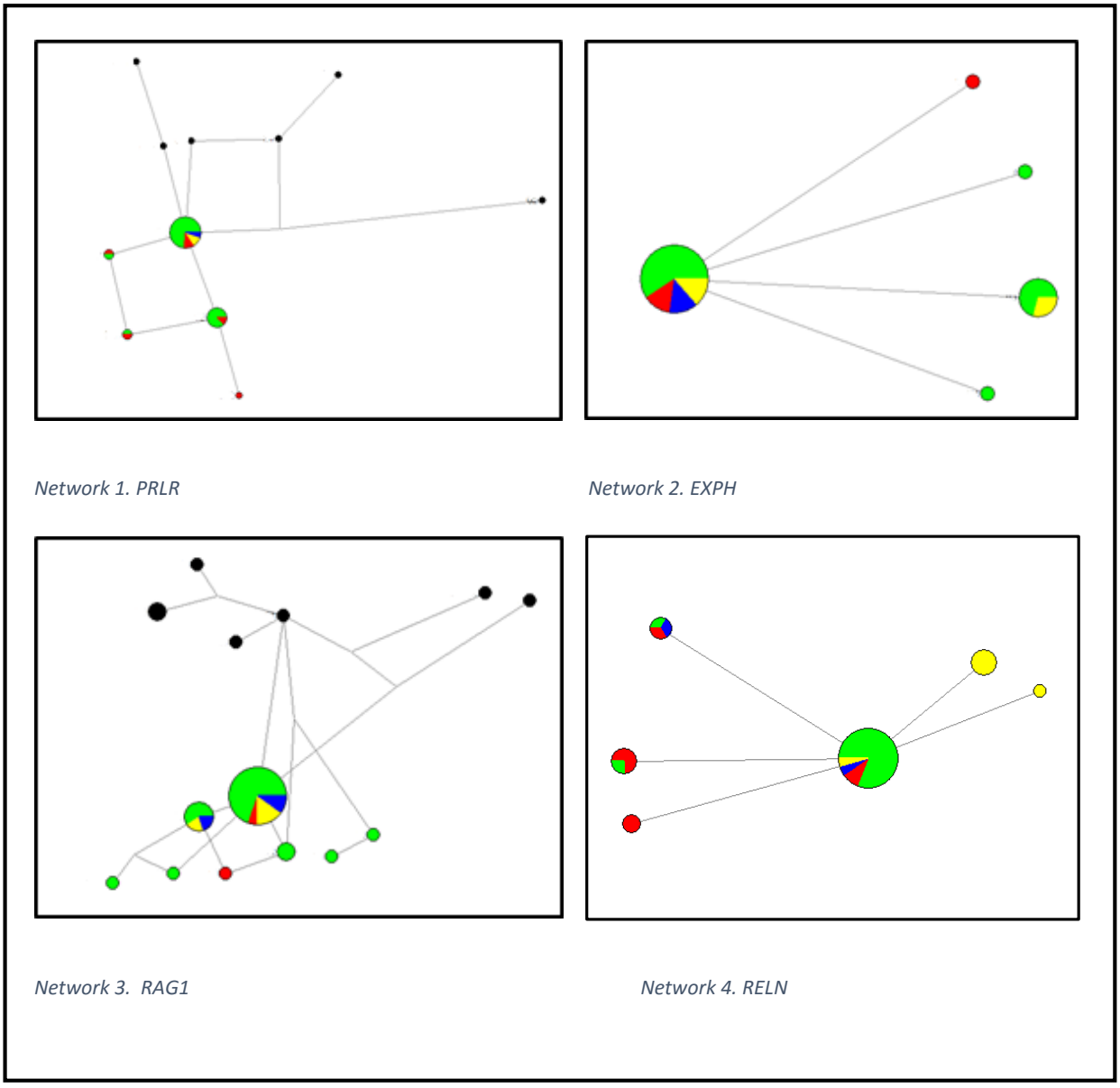


Figure 5 BAPS output showing the numbered samples seperated in to the four identified clades.

Due to the low levels of sequence variability in the nuclear loci, BAPs analyses were run with and without the nuclear data to determine its weight in the phylogenetic analyses. The resulting BAPS outputs were identical, suggesting either the four clades were consistent throughout all of the loci or that the high nucleotide diversity of the mtDNA was drowning out any potentially discordant phylogeographical patterns in the nuclear loci. I used the Fluxus Network program (Bandelt *et al.* 1999) to construct median-joining phylogenetic networks to view relationships among nuclear loci sequences (**Figure 6**).



Network 1. PRLR

Network 2. EXPH

Network 3. RAG1

Network 4. RELN

Figure 6. Phylogenetic networks for the nuclear loci. The size of the nodes correspond to the number of individuals and the networks are coloured coded for ease of compare with the mitochondrial data from BAPS. Green = Individuals BAPS assigned to central clade I, Red = Individuals assigned to central clade II, Blue = Individuals assigned to NW clade and Yellow = individuals assigned to NE clade.

The networks from the nuclear loci show no discernible pattern in a phylogeographical sense. The four main genetic clusters identified from the BAPS analysis are not identifiable in these networks as the colour coding shows. The only network that seems to show some evidence of the four clades is RELN, with the NW clade shown in yellow.

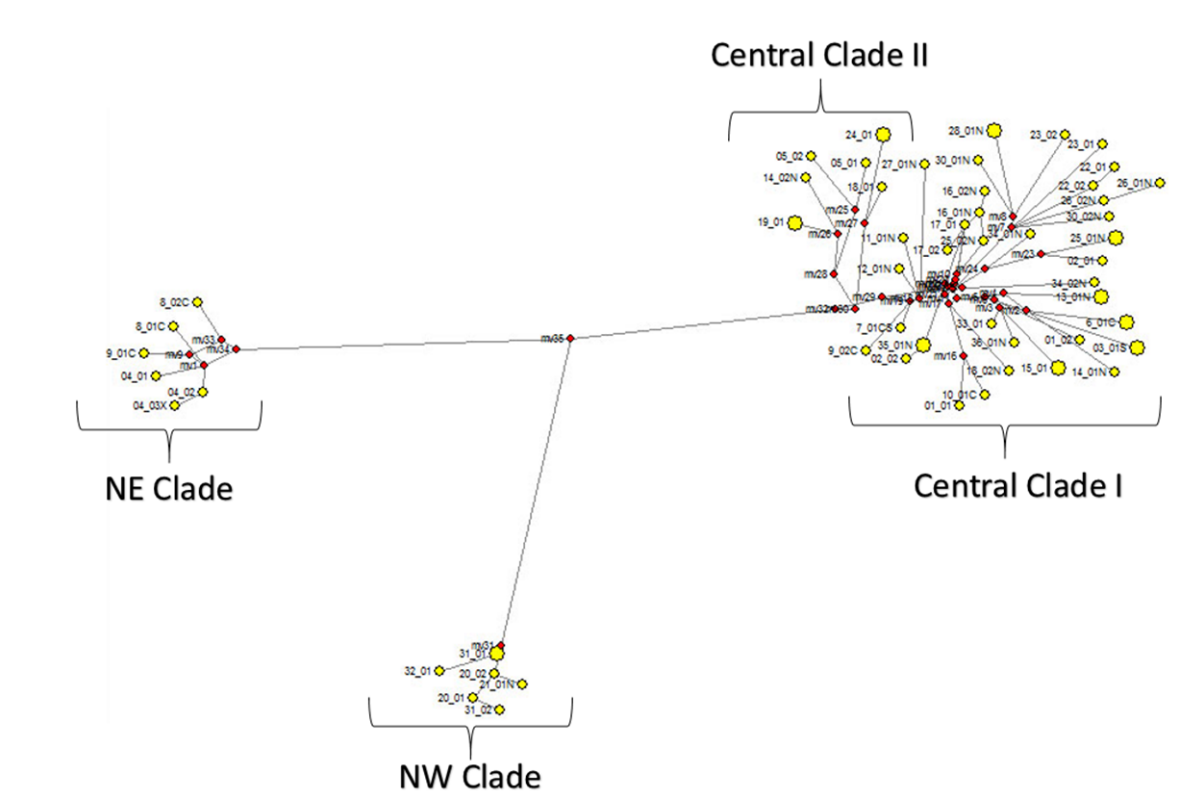


Figure 7 - A haplotype network for the mtDNA sequences. This network shows 4 clades as well as a significant relationship between geographical distributions of clades, similar to those found in the BAPS analysis. Each individual is represented by a yellow node, with the red nodes representing the numbers of mutational steps between individuals.

The haplotype network produced from the mtDNA is markedly different from those produced by the nuclear DNA. Whereas the nuclear networks showed no discernible correlation to the results of the BAPS analysis, the mtDNA network (**Figure 7**) clearly identifies the four genetic clades that were identified by BAPS.

3.3 Bayesian inferences

The Tenerife lineages identified within the BEAST analysis were designated Central clade I, Central Clade II, NW Clade and NE Clade, which were named according to their geographical distribution within Tenerife (**Figure 8**).

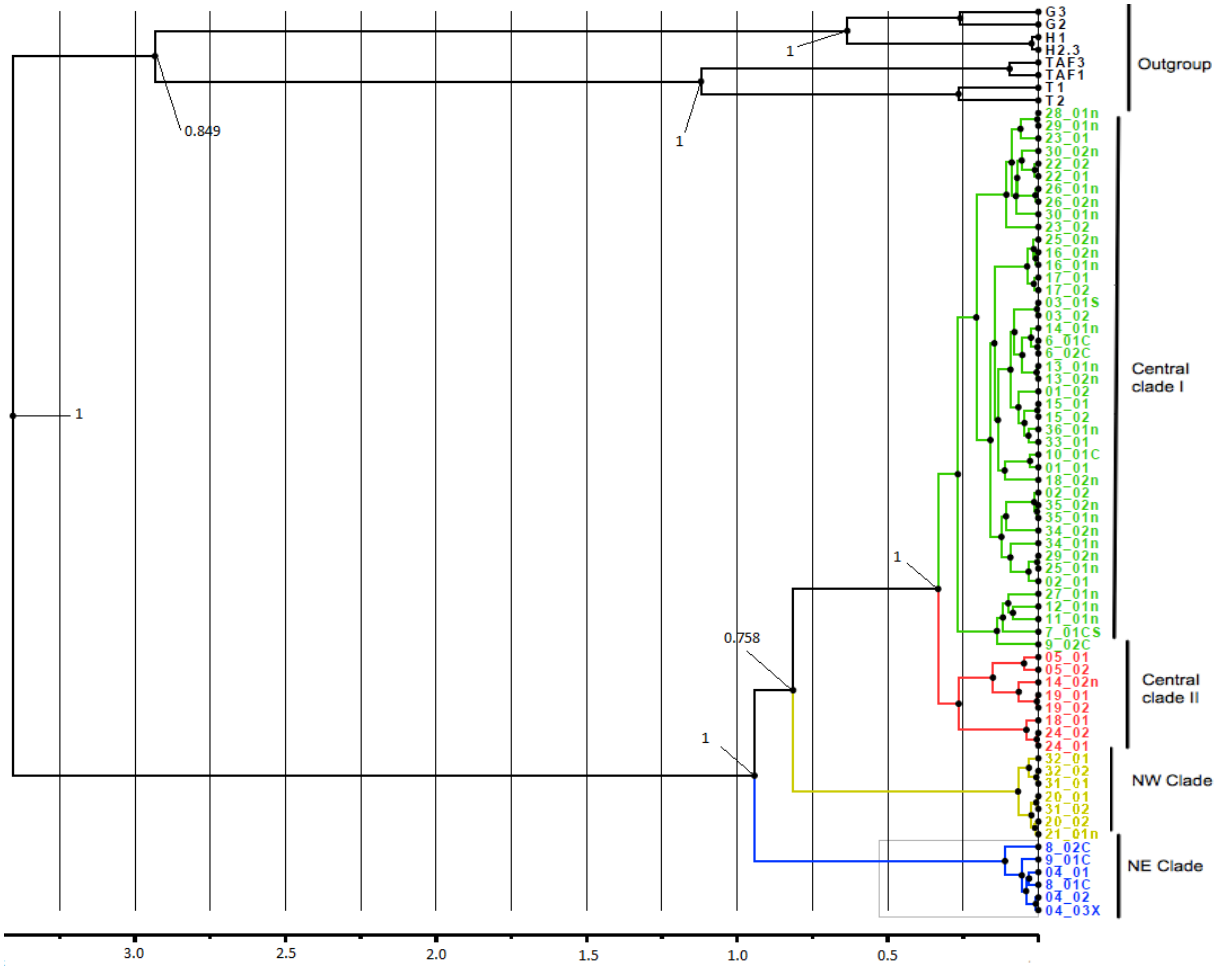


Figure 8 Gene tree based on mtDNA showing the highlighted 4 main clades as well as the out groups. Values shown on tree are support values and the scale on the x axis is in million years.

The most basal node of all of the Tenerife clades, representing a split between the NW, Central I, Central II clades and NE clade, is strongly supported (Bayesian posterior support value (BPS) = 1.0). The Bayesian trees indicate that the NE lineage found at sites 8, 9 & 4 was first to split, with a later event leading to the split between the central clades and the lineage confined to sites 20, 21, 31 & 32 on the very North Western point of the island, although this is less well supported (BPS = 0.758).

Also, the gene tree strongly supports a recent but distinct divergence between two central clades (BPS = 1.0). The large Central clade I comprises of the majority of individuals from sites across the whole central part of the island. This included site 9 where an individual belonging to the NE Clade was also found. Central clade II comprises of individuals mainly located along the northern part of the island, with sites 5 & 14 in the far North East and sites 18, 19 & 24 along the Northern coast (see **Figure 9**).

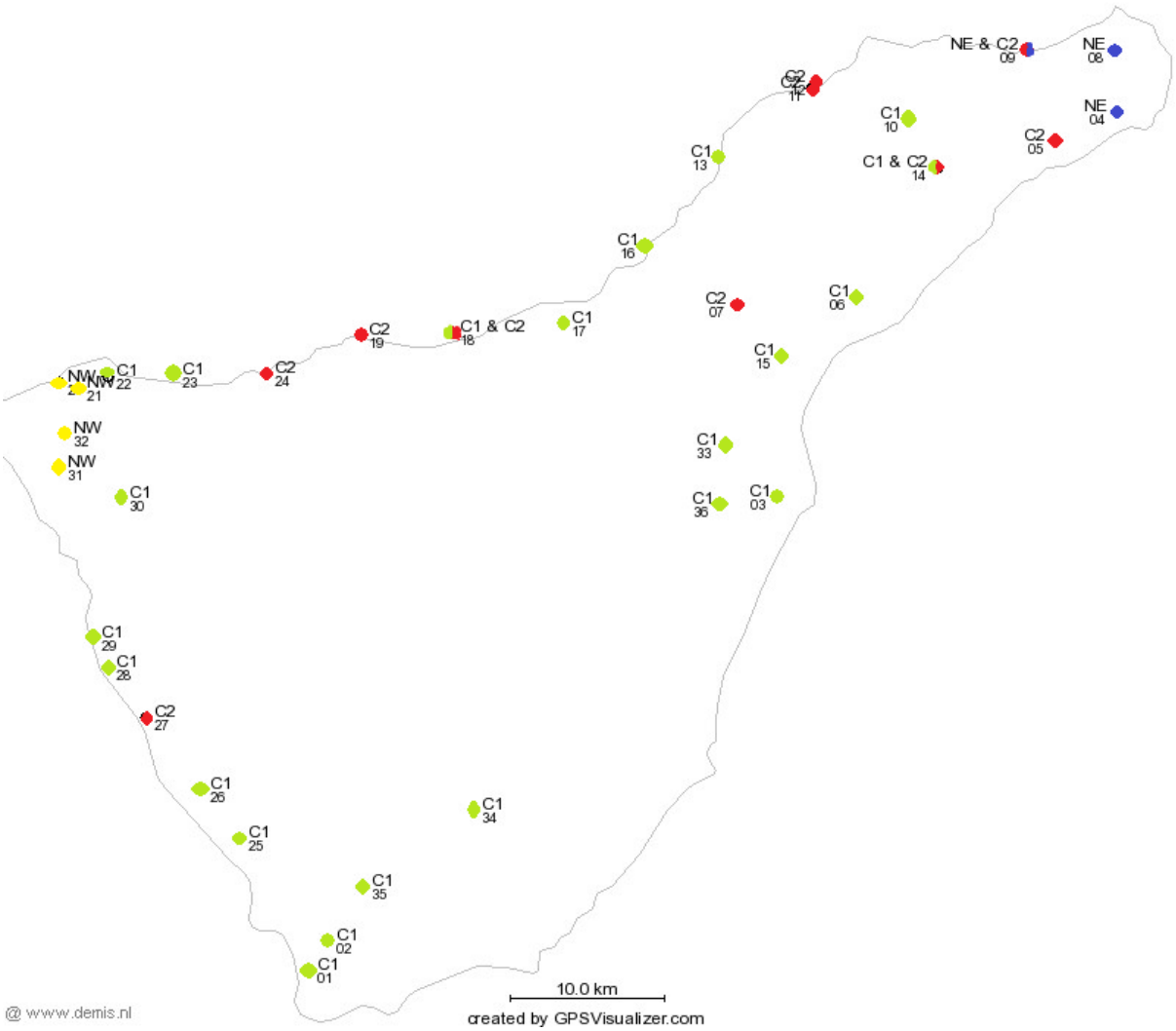


Figure 9. A map of Tenerife showing the locations of the sampling sites. The clades found at each site are labelled (C1 = Central clade I, C2 = Central clade II, NW = North western clade & NE = North eastern clade). The locations are also colour coded for comparison with the BAPS analysis and the gene tree.

3.4 Population substitution rates and divergence times

The nucleotide substitution rates (units are substitutions/site/Ma) differed considerably between partitions. Cytochrome b codons 1 & 2, 0.00383; Cytochrome b codon 3, 0.05510; NADH codons 1 & 2, 0.00786; NADH codon 3 0.05305; tRNA; 0.00737.

The *BEAST analysis indicates that *C. viridanus* diverged from the La Gomera and El Hierro *C. coeruleopunctatus* lineages ~ 4.9 Mya. The North Eastern clade diverged from the other lineages ~ 1.1 Mya with the North Western clade splitting from the central lineages ~ 0.5 Mya. The two central clades on the gene tree show a very recent split of ~ 0.3 Mya.

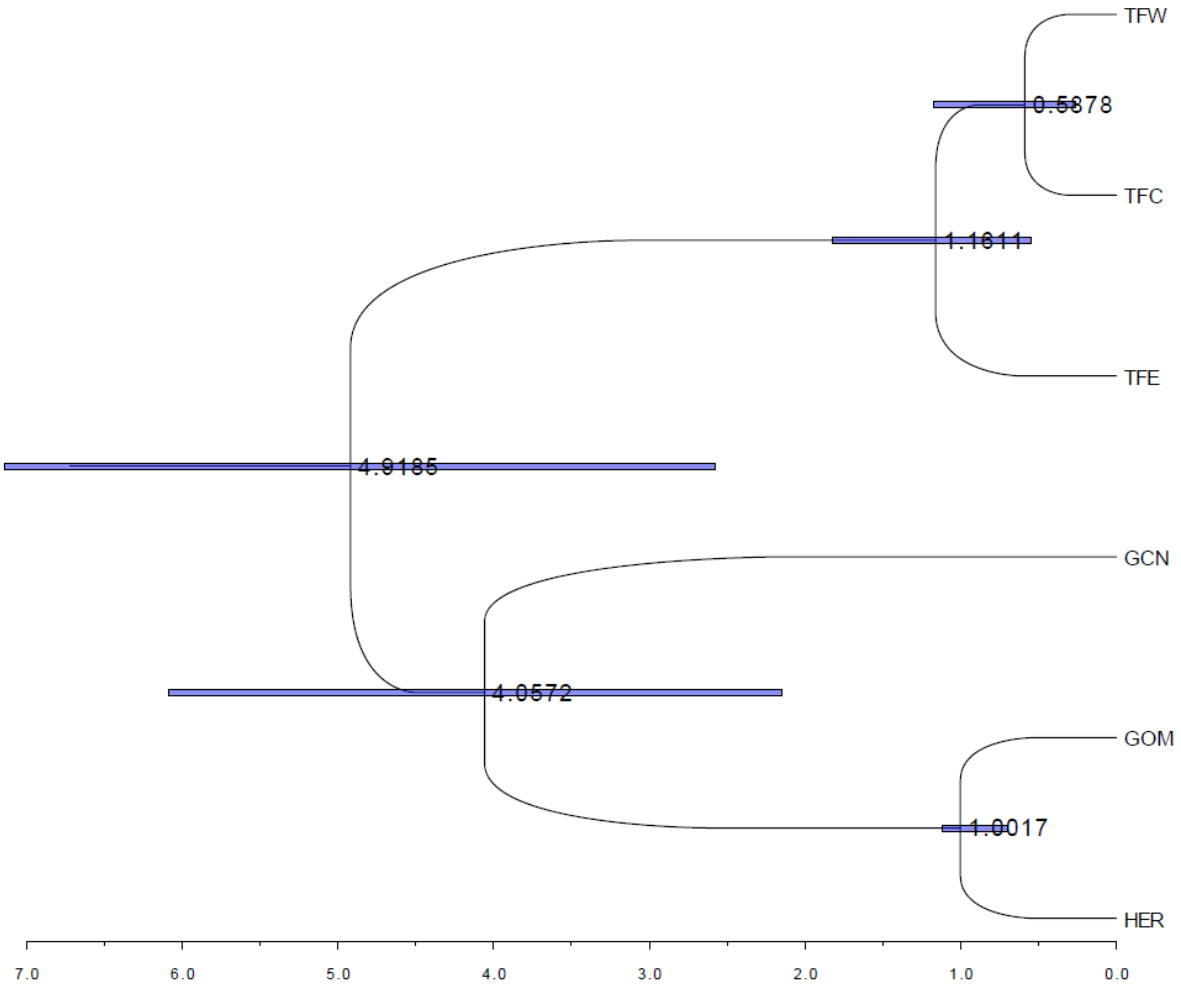
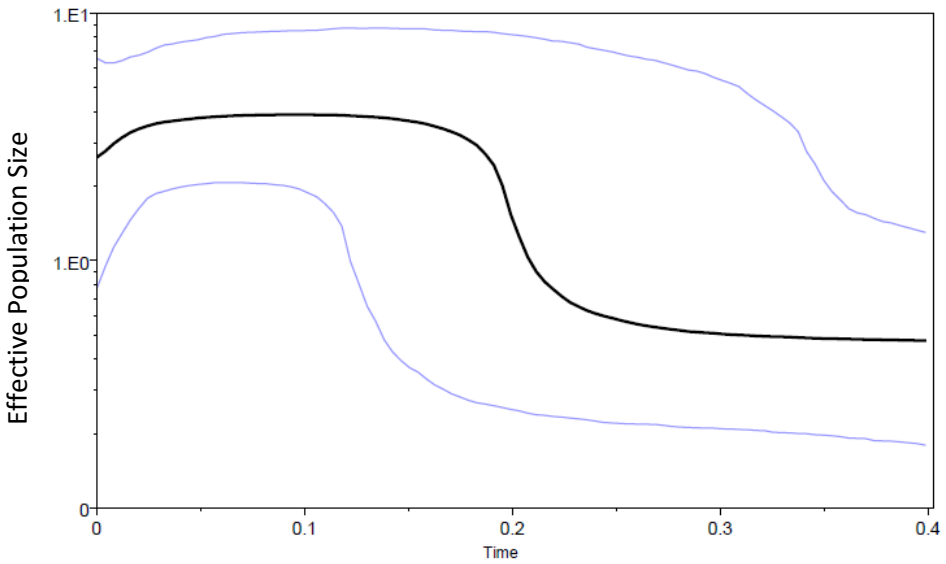


Figure 10 Species tree with the ages of the nodes in million years, bars are 95% posterior intervals.

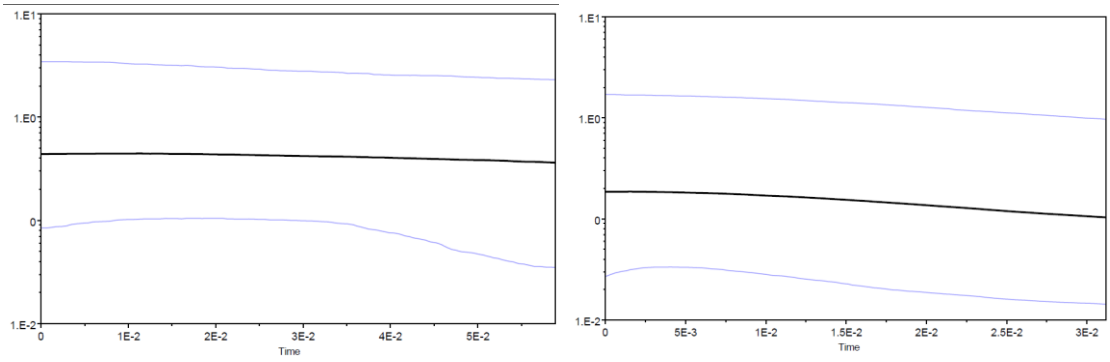
3.5 Bayesian skyline estimation of historic population size

The Bayesian skyline plots for the three phylogeographically distinct lineages provided evidence of a substantial population size increase in the central lineage around 0.2 Mya. There was little evidence of any population changes in the NE and NW lineages, however due to a recent coalescence time for these lineages the BSPs were unable to estimate effective population sizes more than ~.06 Mya (**Figure 11**).

Figure 11. Bayesian skyline plots of the three population groups.



1. *A BSP of the central population showing a substantial increase in the effective population size approximately 0.2 - 0.25 Ma*



2. *BSPs of the northeast (left) and northwest (right) populations showing no substantial increase in effective population sizes in recent history.*

3.6 Species Delimitation

BPP analyses produced different results depending on whether the mtDNA locus was included in the analysis or not. The analysis based on the full data set (mtDNA and the nuDNA) provided strong support for a four species model (posterior probability = 0.951) with speciation probabilities ranging from 0.951 - 1.0 on the guide tree nodes (**Figure 12**). The data set consisting of only the nuclear loci provided strong support for a one species model (posterior probability = 0.80) with weak support for speciation events on the internal guide tree nodes ranging from 0.055 - 0.200 (**Figure 13**). The analyses were consistent across all runs with different prior estimates of Θ and τ, which each run producing similar posterior estimates of both species model and speciation probabilities.

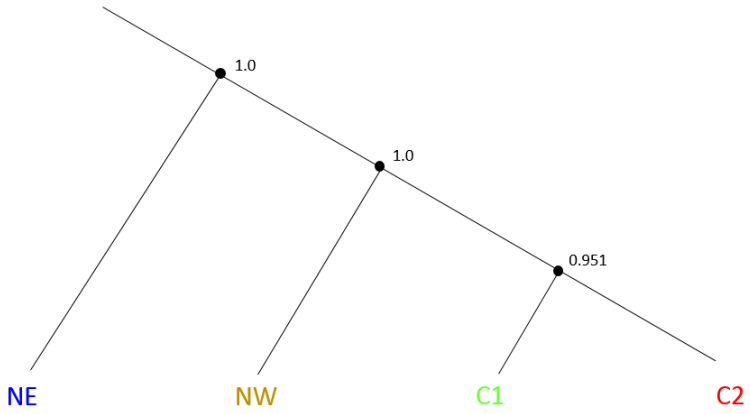


Figure 12 BPP analysis guide tree for the data set containing all loci with estimated posterior probabilities of speciation labelled on the nodes.

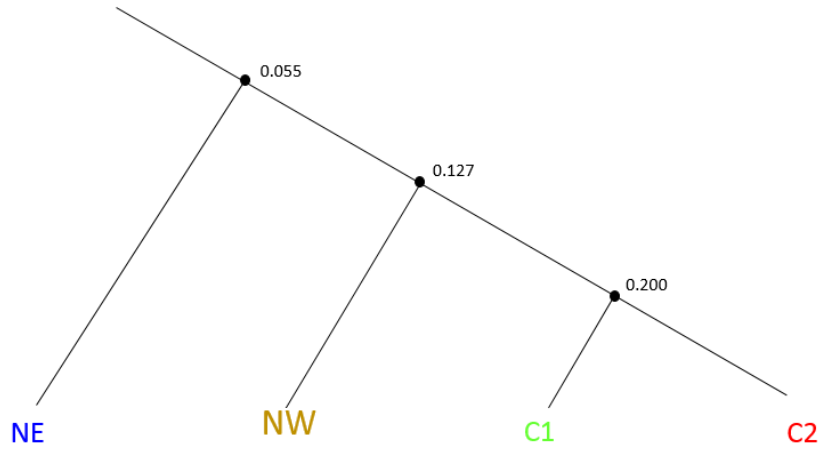


Figure 13 BPP analysis guide tree for the data set containing only nuclear loci with estimated posterior probabilities of speciation labelled on the nodes.

4. Discussion

Using a multi-locus coalescent approach, I discovered some differences from a previous study by Brown *et al.* (2000). The previous study consisted of fewer sampled individuals, less sequence data and more limited, older methods of phylogenetic analyses, of which there are now more advanced alternatives. One of the key differences is that the species tree now shows that the north eastern linage was the earliest to diverge, as opposed to the north western lineage. The new dating methods also appear to suggest the first divergence event occurred approximately 0.3 Ma earlier than previously thought. The dating of the second divergence is ~0.6 Mya which is actually approximately 300,000 years later than Brown *et al.* estimated from their data. One final notable difference is the discovery of a second mt lineage within the central population. This clade is well supported on the mtDNA gene tree and appears to have diverged from the main central clade about ~0.3 Ma, which also potentially coincides with the end of the last major eruptive cycle. However, unlike the other mitochondrial lineages, these two groups show strongly overlapping geographical distributions across the island (**Figure 9**). This does not seem to tie in with any obvious known geological events, unlike the case with the two distinct clades in *Tarentola delalandii* and *Gallotia galloti* across the Guimar valley being linked to a known debris avalanche leading to allopatric populations along the north eastern coast (Gubtiz 2005).

The initial BAPS analysis identified four population clusters within Tenerife, the distribution of two of which seemed to broadly correspond with aspects of the island's geological history, in that there geographical distributions are where the two ancient precursor islands Teno and Anaga were located. However the result of BAPS (**Figure 5**) was identical when performed on all loci together and on mtDNA sequence alone. This suggested that either the nuclear loci were concordant with the mtDNA or the level of sequence diversity in the mtDNA was potentially overpowering any phylogenetically informative information in the nuclear data. The small level of sequence variability in the nuclear loci (between 0.5 - 2.2%) compared to that of the mtDNA (15%) points to the latter being the most likely case. A BAPS analysis on the nuclear DNA loci alone, provided no clear evidence of four clades as shown from the mtDNA results and so haplotype networks where constructed on each nuclear loci individually to examine if any singular nuDNA locus was concordant with the mtDNA data.

The haplotype networks constructed from the nuclear loci (**Figure 6**) clearly show significant discordance with the population groups discovered using BAPS, again strengthening the suspicion that lack of informative sites in the nuclear loci was leading to mtDNA signal being significantly overpowering. The discordance with BAPS and the highly polyphyletic appearance of the haplotype networks suggests that there may be incomplete lineage sorting of the nuclear loci within the population groups on the island.

Lineage sorting is the process by which gene lineages become fixed within a population so that all alleles within a species sort to a single ancestral allele for that species. Complete lineage sorting of alleles within a species will result in any sampling of those alleles to lead to a reciprocally monophyletic gene tree (Avise 1989; Maddison 1997). On the other hand incomplete lineage sorting is the process by which ancestral polymorphisms can persist through species diverged up to several million years. Several factors can increase the amount of time taken for lineage sorting to complete. Population size and generation times are thought to be quite influential, with larger populations and longer generation times protracting the time taken for complete lineage sorting to occur. Incomplete lineage sorting can be especially problematic for populations with fairly recent divergence times (Knowles 2002, Zink & Barrowclough 2008) In this case *BEAST analyses on the mtDNA data estimated the earliest divergence at only 1.16 Mya and the most recent at just 0.6 Mya. Hence divergence was recent in this species and so there may have not been enough time for lineage sorting to complete. The lower mutation rates in nuDNA (Brown 1983) and a larger effective population size compared with mtDNA mean that it can take up to four times longer for lineage sorting to occur. This is most likely why lineage sorting appears to be complete in the mtDNA but not in the nuclear loci. This case may be a good example of the mtDNA being a leading indicator in the phylogeny and the nuDNA not necessarily being discordant due to factors such as introgression, but merely because the nuDNA needs more time to resolve on the same species tree. Introgression is an unlikely cause for the observed discordance between the loci because the polyphyletic alleles are present in individuals situated all over the island and not concentrated close to contact zones as one would expect if introgression were the case (See **Figure 9**).

Due to the effects of incomplete linage sorting, the trees constructed as well as the divergence times estimated via phylogenetic analyses can become uninformative within a species (Doyle

1992, Posada & Crandall 2001). That was the case in this study, *BEAST analyses that included all loci gave poor posterior results, with many runs struggling to reach stationarity leading to wildly inaccurate estimations of the divergence times between populations on La Gomera and El Hierro (which is known to have occurred no later than the emergence of El Hierro 1.12 Mya). This was the case even when using the simplest substitution & clock models for each of the nuclear loci. It is likely that the lack of sequence variability combined with the effects of incomplete lineage sorting in the nuDNA led to *BEAST struggling to estimate coalescent times from the data. When the *BEAST analysis was run on the mtDNA data alone the posteriors (specifically for the La Gomera, El Hierro divergence times) were much more realistic and more closely resembled previous estimates (Brown *et al.* 2000). Therefore the identification of clades and divergence times for *C. viridanus* were taken from Bayesian inferences of coalescent times made from the mtDNA. Despite various cautionary papers on the use of mtDNA alone to estimate phylogenies (Shaw 2002, Ballard & Whitlock 2004, Rubinoff & Holland 2005) many studies still do (Kozak *et al.* 2005, Lemmon *et al.* 2007, Wilson *et al.* 2009). A phylogeny based on a single mtDNA loci may still provide a reasonable estimate of the overall species tree (Wiens *et al*. 2006). Even when contructing a phylogeny from multiple loci when there is little variability in the nuDNA data, as is the case here, the phylogeny may still be dominated by the phylogenetic signal from the mtDNA due to its greater number of variable sites. Although the lack of sequence variability in the nuDNA had a frustrating effect on the reliability of the BAPS and the *BEAST analyses, discovering evidence of incomplete lineage sorting is a significant finding in and of itself, because it provides additional evidence towards the recent divergence times that *BEAST estimated from the mtDNA data.

The phylogenetic inferences made from the *BEAST analysis appears to show a clear link with the geological history of Tenerife and the cladogenesis found within *C. viridanus* on the island. The evidence of four clades, two located centrally, one in the NW and one in the NE appear to fit with what is known about the geological history of the island. Potassium-Argon dating methods of rocks across Tenerife have found evidence of three ancient areas; Anaga in the North East, Teno in the North West and Adeje towards the South West of the island. These areas have been aged at approximately 6.5 Ma, 7.5 Ma and 11.6 Ma respectively (Ancochea *et al*. 1990).

The distributions of three out of the four clades shown in the species tree seem to fit with the

idea of there being populations on these previously separate precursor islands. Individuals belonging to the North Western and North eastern clades are found nowhere else but these locations on the island associated with Teno and Anaga. Meanwhile the two central clades having a wide distribution across Tenerife seem consistent with the idea they had an association with the ancient island Adeje in the southwest and have since undergone a considerable post colonisation range expansion following a recent bottleneck around 300,000 years ago (Brown *et al.* 2000).

The earliest divergence of all of the Tenerife clades, represented a split between the NW, Central I, Central II clades and NE clade and is strongly supported. Tenerife has been the site of many violent volcanic events throughout its entire history. These volcanic cycles originally formed the main island, with the central Cañadas edifice uniting Teno, Anaga and Adeje between 3.5 and 2.7 Mya and further eruption cycles occurring between 2.5 - 1.4 Mya and 1.1 - 0.2Mya (Ancochea, 1999).

Colonisation is thought to have taken place ~4 Mya (Brown & Pestano 1998) and despite the precursor islands probably being joined at the beginning of the first eruptive cycle approximately 0.5 Ma later, the first divergence in *C. viridanus* populations on the island according to the *BEAST analysis occurred ~1.16 Mya between the central and north eastern lineages. The north western lineage then diverged from the central population approximately 0.6 Mya. This seems almost counterintuitive, as divergence between the populations would be expected to occur when the populations became isolated, i.e. before the precursor islands joined. However, as previously highlighted, Tenerife's geological history is a complex one due to the three prolonged eruptive cycles and any number of vicariance events could have occurred over this time period. For example Bayesian skyline plots (BSPs) estimating any changes in the effective population sizes of the three lineages show a substantial increase in the central population group between 0.2 – 0.25 Mya, which appears to coincide with the end of the last eruptive cycle (Guillou *et al.* 2004) suggesting that volcanic activity may have kept the *C. viridanus* populations confined to specific areas of Tenerife until this time. After which the central population associated with the ancient precursor island Adeje proceeded to expand across the rest of Tenerife.

Studies on morphology have found significant differences in body dimensions and scalation across the island that is correlated with the northern mesic and southern xeric environments

(Brown *et al.* 1993), but these are not reflected in the molecular data. Giving there are no seemingly obvious morphological differences that could lead to a reproductive barrier, between the expanding central clade and the two more isolated clades associated with Teno and Anaga

The attempted species delimitation in this study produced incongruent results when BPP analyses were ran on a data set containing all loci compared to that of a data containing only the nuclear loci. Again this is more evidence of the mtDNA sequence data having a much higher level of diversity than the nuDNA data and therefore overpowering the posterior on number of species. The results of the BPP analysis from the data set containing all loci estimated an overwhelming support for a four species model, suggesting that there is significant evidence to delimit the four mitochondrial clades into separate species. However it is extremely unlikely that the two central clades could represent actual evolutionary lineages. Their geographical distribution alone, along with no consistent morphological differences between the two central clades make the hypothesis that they are separate species distinctly improbable. Whereas conversely, the results from the nuclear loci present a high level of support for a one species model. This conclusion not only again highlights the lack of diversity in the nuclear sequence data, but would also make more sense from a taxonomical point of view. Due to the discordance between the two results however, the robustness of either conclusion on the species delimitation of *C. viridanus* is highly questionable.

Species have traditionally been identified and described using morphological traits. However morphological traits such as coloration, feeding or sexual morphology may be undergoing convergent evolution as they are under similar selection pressures, so identification of species using morphology alone can be misleading. Another problem of using purely morphological traits in species classification is that it may fail to identify the more cryptic species and therefore underestimate the number of species present (Yang & Rannala, 2010). Over the last decade, the use of molecular methods to identify species has become a standard, but it is not without its problems. BPP as a tool for species delimitation is known to have some short comings. Firstly, its dependence on the accuracy of the guide tree. It requires a user submitted guide tree topology which the program then uses to guide the reversible jumps in the Markov chain. It has been demonstrated that any inaccuracy in the guide topology can lead BPP to

delimit each of the putative lineages, due to the artificial increase in genetic distance between sister lineages (Leache & Fujita 2010). In this case a guide tree was selected with both central clades specified as hypothetical distinct species,to provide a straw man proposal for the accuracy of species delimitation in this case. If BPP selected a four species model (as it indeed did) in this case, then the suitability of the data for a robust estimation of species delimitation would be called in to question. It has been shown that species delimitation probabilities can be difficult to estimate from guide trees with a low level of species divergence (O'Meara 2010) and this could be the problem faced here. The low levels of divergence in the nuclear loci compared to the relatively high levels of divergence in the mtDNA are leading BPP to falsely identify the mitochondrial clades as distinct species.

One other common problem that can lead to potential shortcomings in species delimitation is limited sample size. It has been suggested that at an absolute minimum, researchers should collect at least 10 samples from all putative lineages in order to obtain a high probability (>90%) of sampling the deepest coalescent events in each population, insuring that the most meaningful genetic variation, in aid of species delimitation, is sampled (Carstens *et al.* 2013). So in this case further sampling of the north western and north eastern clades may be necessary if a robust attempt at species delimitation is to be achieved.

Although the difference in the BPP analyses between the two data sets makes it difficult to accept either species delimitation model as accurate, it has been shown that a cautious approach is the best approach to take and failing to delimit species is preferable to falsely delimiting clades that do not represent actual evolutionary lineages (Carstens *et al.* 2013). For example a recent study by Salter *et al.* (2013) used different methods to delimit between 3-18 lineages of trapdoor spiders along the west coast of North America. They interpreted this incongruence in a conservative manner and only recognised 3 of these lineages as species. So for the time being it seems the most prudent thing to do is accept the one species model in the case of *C. viridanus* on Tenerife.

One other cautionary note on the subject of species delimitation in this case is that morphological differences should also serve as a basis for taxonomic inferences. In instances such as this were a north/south variability in morphological traits such a tail pigmentation is incongruent with phylogenetic variation, extra care should be taken in species delimitation (Leliart *et al.* 2009, Barrett & Freudenstein 2011).

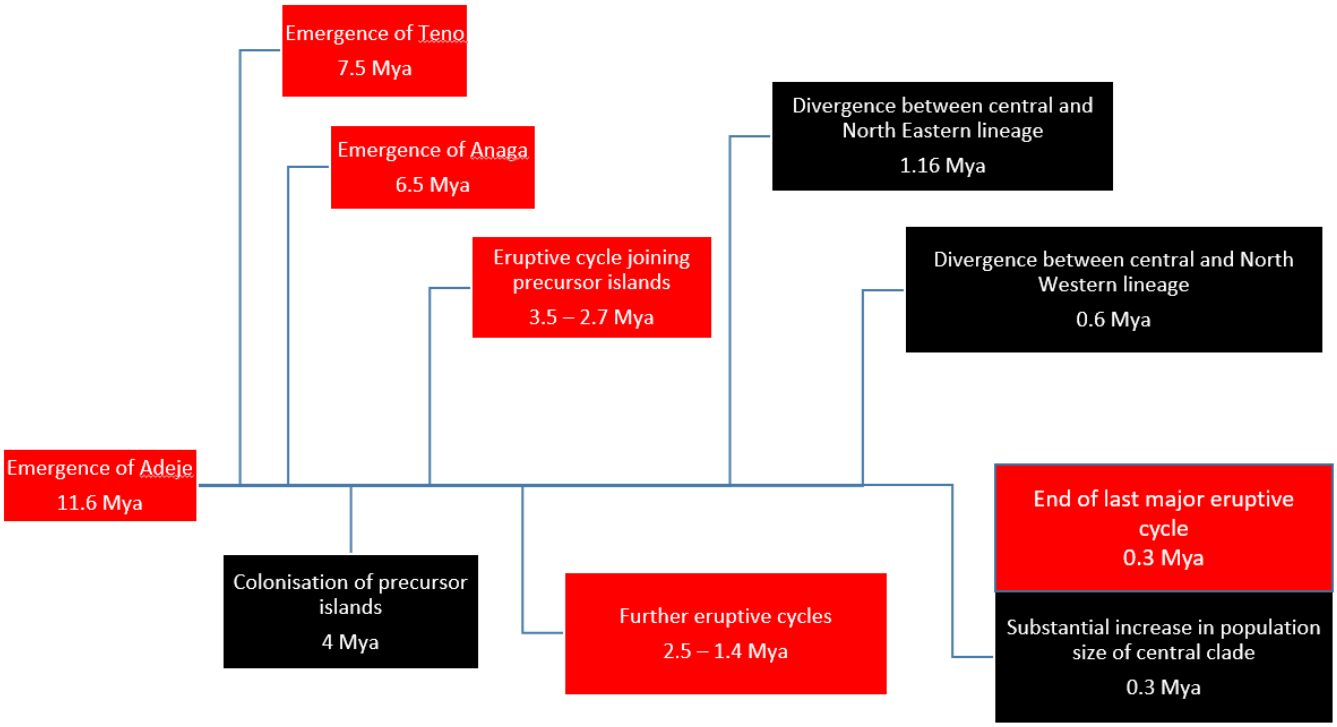


Figure 14. Graphic representing a timeline of events on Tenerife. The red boxes represent historical geological events and the black boxes represent phylogeographical events. (Mya = Million years ago).

Overall this study has shown that an investigation of the Tenerife skink *Chalcides viridanus* using a more comprehensive sampling method and more advanced techniques was essential in order to gain a more robust insight into the species phylogeographic history. While much of the work re-emphasises already established ideas, this study has enabled an increased resolution of intra-specific relationships within *C. viridanus*, providing statistical support for how it's linked to the geological history of Tenerife.

Despite the importance of a multilocus coalescent approach in the accurate resolution of species trees in the field of phylogeography, this study stands as a reminder that nuclear loci do not always infer an accurate species history in the case of recently diverged lineages. Complicating factors such as lack of sequence diversity and incomplete lineage sorting in nuclear loci can often obfuscate the true phylogeographic history of a species.

Acknowledgements

I would like to thank my supervisor Professor Richard Brown, without whom this thesis would not have been possible. I was offered a great deal of guidance in this field of research and unparalleled support, with infectious enthusiasm, during all stages of this research. His input with the use of analytical techniques in particular was invaluable and with his help I was able to overcome several significant problems in this stage of the project.

My second supervisor, Dr Jerry Bird, was incredibly helpful in terms of getting me settled in the lab and always ensured I had the materials I needed to complete my work. Unofficial lab technician Aire (Thanawa Tejangkura) was an invaluable tutor in molecular lab work. She taught me many of the molecular techniques I used in this project and her vast experience in the lab helped me solve many problems for the time she was around.

I would also like to thank my friends and family for their support and encouragement, as well as my part time employers for their patience and understanding with allowing me time to complete this MPhil project.

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