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# THE EFFECT OF THE GEOLOGICAL HISTORY OF THE CYCLADES ON THE PATTERN OF DIVERSIVICATION IN VIPERA AMMODYTES

by

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# ABSTRACT

## THE EFFECT OF THE GEOLOGICAL HISTORY OF THE CYCLADES ON THE PATTERN OF DIVERSIVICATION IN VIPERA AMMODYTES

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The Cyclades are a group of islands found in the Aegean Sea. The islands have long been a popular research destination due to the interesting patterns of diversification found in the terrestrial animals that inhabit them. Even though these patterns of diversification have been researched, studies have failed to address the recurrent nature of this pattern or offer possible explanations for its occurrence. To further investigate this phenomenon, we performed ddRADseq on 38 samples of *Vipera ammodytes* from the Cycladic islands. After the sequences were processed, we performed a Structure analysis and built a maximum-likelihood phylogenetic tree using RAxML. Our results indicated that there is a significant difference between the populations found on the northern and southern islands. Furthermore, we propose that this pattern of diversification is due to

glacial and interglacial cycles that lead to the continuous formation and disappearance of land bridges between the islands.

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# CHAPTER 1

# INTRODUCTION

The Cyclades are a group of islands located off the coast of Greece in the Aegean Sea. The islands have long been a popular research destination due to unique patterns of diversification found in the terrestrial animals that inhabit them. Multiple factors could have affected the diversification of these animals, the biggest being the geological shifts and changes that took place over the last couple of million years.

Starting in the late Miocene (5.3 MYA), tectonic events lead to the separation of the Cyclades from continental Greece, Crete and the Dodecanese islands (Anastasakis *et al.* 1990). During the early Pliocene (5.2 MYA) the islands were then separated into northern and southern Cycladic islands (Roussos 2015), with further fragmentation taking place during the Tertiary and Quaternary. This further fragmentation was likely due to sea level changes caused by glacial and interglacial cycles that lead to the formation and disappearance of land bridges connecting the islands (Sagonas *et al.* 2014). These occurrences make the Cyclades the perfect location to study how geological changes affect the diversification of species. The same can also be said for the opposite. Through conducting genetic studies on the different taxa found on these islands, we can obtain a better understanding of their colonization patterns and leverage these patterns to further understand the geological history of the islands.

One such example is the recurring pattern of a northern and southern island split between different populations of the same species. This unique pattern of diversification has been well studied in a variety of species including *Laudakia stellio*, *Podarcis milensis* and *Mesobuthus gibbosus* to name a few (Parmakelis *et al.* 2006). We also have reason to believe that the same could be possible for *Vipera ammodytes*. The specie is widespread across the islands and mainland, and a previous study indicated the possibility for unique northern and southern island group clades (Ursenbacher *et al.* 2008). However, this study only used four samples, two from the southern and two from the northern Cycladic islands, and thus more extensive sampling is needed to fully understand the relatedness of individuals within and between these island groups. Here, we utilize dense sampling and ddRADseq data to demonstrate the presence of distinct northern and southern island clades in *Vipera ammodytes*, and provide a context of historical geological changes in the Aegean Sea that may underlie this recurrent pattern of divergence. This conserved pattern of divergence across the Cycladic islands argues for considering northern island and southern island population groups as evolutionarily significant units, both in *V. ammodytes* and other Cycladic species that likely diverged in a similar manner, for future conservation efforts in the area.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Geography and Biogeography of the Cyclades

The Cyclades consists of a group of islands located in the Southern part of the Aegean Sea and are well known for their indigenous diversity (Parmakelis *et al.* 2006). The islands can be divided into different subgroups, two of which consist of northern and southern Cycladic islands. The northern islands consist of Andros, Tinos, Syros, Rinia, Delos and Mykonos, while the southern islands consist of Despotiko, Antiparos, Paros, Naxos, Silkinos, Ios, Iraklia and Ano Koufanissi (Roussos 2015).

During the late Miocene (Serravallian to the Messinian age) tectonic events lead to the separation of the Cyclades from Continental Greece, Crete and the Dodecanese islands (Anastasakis *et al.*1990). At the beginning of the Pliocene, the Zanclean flood lead to the further separation of the islands into the northern and southern Cycladic islands (Roussos 2015). Additional fragmentation within the northern and southern island groups continued during the upper Tertiary and Quaternary due to sea-level changes caused by glacial and interglacial cycles that lead to the formation and disappearance of land bridges (Sagonas *et al*. 2014). The degree of connectivity between these islands depended on the magnitude of sea level change that occurred during these cycles, the first of which took place at the end of the Pliocene (3.6 MYA). More severe changes in sea level were seen during the Pleistocene (2.7 MYA), with the most severe taking place within the last one million years as the glacier cycles intensified (Roussos 2015).

Not only have geological and climatic factors have an effect on the Aegean, but also humans who have been living on the islands for the past 10,000 years. Numerous ecosystems on the islands are currently affected and under threat because of changes in their habitat due to the transportation of animals (Roussos 2015). Due to this unique pattern of diversification between populations of different species found on the islands, and also possibly in *Vipera ammodytes*, it is vital that we implement more effective means to protect these populations. Only through genetic studies of these Cycladic populations will we be able to get a clear picture of the geological history of the islands and how past events helped shape them (Ursenbacher *et al.* 2008).

### 2.2 *Vipera ammodytes*

Mammals, birds, fishes, amphibians and invertebrates have been the subject of many phylogeographic studies done in Europe. Snake species on the other hand have been far less researched and not a lot of phylogeographic data are available on them. Snakes of the genus *Vipera* have been known in Europe since the early Miocene. Fossils found dating back to this period belonged to the "*Vipera apsis* complex", which includes *Vipera aspis*, *Vipera ammodytes* and *Viper lataslei.* These three species can currently be found in many parts of southern Europe, with *V. ammodytes* being the most widely distributed (Ursenbacher *et al.* 2008).

The most probable origin of the *Vipera ammodytes* complex was determined to be in the western Balkans between Albania and Croatia. During this period the snakes were greater in size and their diet included mammalian and amphibian prey that could be found on the mainland. Due to changes in the landscape during the Miocene these lineages began to diverge as the animals became isolated in the Cyclades. Further diversifications took place during the early Pliocene as the Zanclean flood separated the islands into northern and southern Cycladic islands. Sea-level changes in the Mediterranean during the Pleistocene also contributed to diversification between populations found on smaller island clusters within the northern and southern Cycladic groups. The populations currently found on the islands are smaller in size and their diet shifted to small lizards and centipedes (Roussos 2015).

Genetic studies on the different taxa found on these islands could provide unique insight into their colonization route and how past geological events affected them (Ursenbacher *et al.* 2008). Previous studies have shown that it appears that the snakes did not swim between islands, and that the only possible way for gene flow to occur would be via the formation of a land bridge connecting them (Roussos 2015).

### 2.3 Pattern of Diversification

Through studying diversification among other terrestrial animals found on the islands, a recurring pattern seemed to emerge. In a study done on lizards (*Laudakia*) the authors set out to investigate the effects of geological processes on the genetic differentiation and speciation within the populations. Through comparing two different populations, *L.s. stellio* found on the northern islands of Mykonos and Delos, with *L.s. daani* found on the southern islands of Paros and Naxos, it was seen that the two differed extensively. Not only were they different in morphological and chromatic characteristics, but also in displayed behavior. Along with differences between the sub-species, the authors also observed changes in morphology, display structure and head color within the different sub-species (Bramman *et al.* 2010).

This same pattern of divergence was also seen in other species that inhabit the islands. The Milos wall lizard (*Podarcis milensis*) and a scorpion specie (*Mesobuthus gibbosus*) show this same pattern of diversification between populations found on the northern and southern Cycladic islands (Parmakelis *et al.* 2006).

Island populations of snakes found in other parts of the world have illustrated their ability to change and adapt after a divergence from a mainland population. This change and adaptation is often characterized by a change in body size, shape, coloration and predatory and reproductive behaviors (Roussos 2015). A study previously done on *Vipera ammodytes* in the Cyclades revealed that there was a significant difference between populations found on the mainland, as well as populations found on the northern and southern islands. The author explained that this could be due to changes in the sea level that lead to the populations being isolated (Ursenbacher *et al.* 2008). During this study however the author only used four samples, two from the southern islands and two from the northern islands, to study the divergence in the Cyclades. With this preliminary data suggesting the same pattern of diversification in *Vipera ammodytes*, more sampling of the populations found in the Cyclades are necessary in order to get a more complete picture.

### 2.4 ddRADseq

The samples of *Vipera ammodytes* that we used in our study were obtained from various museums and also from animals caught in the wild. Due to the amount and limited availability of our samples we decided to use the Next Generation Sequencing (NGS) method, ddRADseq, to obtain our sequences and get a good sense of the genetic diversity in the specie without sequencing the whole genome.  ddRADseq uses a two-enzyme double digest after which precise size selection takes place. During size selection, regions that are either very close or very far from the restriction enzyme recognition sites are excluded. This allows us to obtain libraries containing only the specific fragments close to our target size. This method also eliminates several steps in which high amounts of DNA could potentially be lost, allowing us to create ddRADseq libraries with starting DNA of 100ng or less (Peterson *et al.* 2012).

# CHAPTER 3

# METHODOLOGY

The samples that we used for our study were obtained from various museums (Goulandris Natural History Museum and Natural History Museum of Crete) as well as from individuals caught in the wild (total of 100 individuals from the mainland and Cycladic populations). First we extracted the DNA from 38 samples of *Vipera ammodytes* and one sample of *Vipera graeca* (to be used as an outgroup) by performing a standard Phenol-Chloroform Isoamyl Alcohol DNA extraction. After the DNA was extracted, we performed a standard Qubit assay on the samples to determine the concentration of the DNA. If the concentration was not sufficient (<500 ng/uL), PCI was performed again until a desirable concentration was obtained. The samples were then cleaned using a standard Serapure bead cleaning procedure, and afterwards a Bioanalyzer (Agilent, Santa Clara, CA, USA) was used to determine the concentration and the size of the DNA fragments present in the samples. We generated double-digest restriction-enzyme associated sequence libraries for all 39 samples. DNA was digested using rare (SbfI, 8 bp recognition site) and common (Sau3AI, 4 bp recognition site) cutting restriction enzymes. Indexed adapters containing unique molecular identifiers were ligated to the ends of digested fragments, and samples were subsequently pooled into groups of 8-12 samples (Schield *et al.* 2015). These pooled samples were size selected for a range of 575 – 655 bp using the Blue Pippin Prep (Sage Science, Beverly, MA, USA), and were amplified using PCR to attach appropriate flow-cell binding sequences and a second identifier index specific to each pool of samples.

These 8-12 sample pools were combined in equimolar amounts into a final pooled library and sequenced using 150 bp paired-end reads on an Illumina HiSeq.

ddRADseq data was processed using the Stacks pipeline (Catchen *et al.* 2013). In brief, PCR clones were identified and removed, sequencing adapters were trimmed from the beginning of the sequence reads, individual samples were demultiplexed using the individual-specific unique molecular identifiers, and overlapping reads across individuals were used to identify *de novo* loci and call single nucleotide polymorphisms (SNPs) within those loci. Samples with high missing data (>80%) were removed for all subsequent analyses. A maximum-likelihood phylogenetic tree was constructed using RAxML (Stamatakis 2014) with the *V. graeca* sample as an outgroup. We also used STRUCTURE (Evanno *et al.* 2005) to infer population structure across 27 island individuals. STRUCTURE Harvester (Earl and von Holt, 2012) was used to determine the most highly supported number of distinct genetic groups represented within the data, and STRUCTURE PLOT (Ramasamy *et al.* 2014) was used to visualize the bar plot generated in STRUCTURE.

# CHAPTER 4

# RESULTS

## 4.1 Sequencing Overview

The following samples obtained in the Cyclades and on the mainland, Greece, were used in our experiment:

Table 4.1: *Vipera ammodytes* and *Vipera graeca* Samples used in the experiment. We gathered information related to Location, Sex, Age and SVL (Snout to Vent Length) for each Sample.

| **Sample ID** | **Taxonomy** | **Location** | **Sex** | **Age** | **SVL** |
|---|---|---|---|---|---|
| VA0001 | Vipera ammodytes meridionalis | W. Syros, Cyclades, Greece | F | NaN | NaN |
| VA0002 | Vipera ammodytes meridionalis | S. Rinia, Cyclades, Greece | F | NaN | NaN |
| VA0005 | Vipera ammodytes meridionalis | S. Evia, Greece | F | NaN | NaN |
| VA0006 | Vipera ammodytes meridionalis | C. Syros, Cyclades, Greece |  | NaN | NaN |
| VA0019 | Vipera ammodytes meridionalis | W. Ano Koufonissi, Cyclades, Greece |  | NaN | NaN |
| VA0038 | Vipera ammodytes meridionalis | NC. Naxos, Cyclades, Greece | M | AD | 550 |
| VA0051 | Vipera ammodytes meridionalis | S. Sikinos, Cyclades, Greece | F | AD | NaN |
| VA0073 | Vipera ammodytes meridionalis | SW. Naxos, Cyclades, Greece | NaN | NaN | NaN |
| VA0076 | Vipera ammodytes meridionalis | Vari, Attica, Greece | F | JUV | 172 |
| VA0077 | Vipera ammodytes meridionalis | SE. Paros, Cyclades, Greece | M | JUV | 145 |
| VA0090 | Vipera ammodytes meridionalis | SE. Paros, Cyclades, Greece | F | AD | 185 |
| VA0091 | Vipera ammodytes meridionalis | E. Paros, Cyclades, Greece | M | AD | 268 |
| VA0093 | Vipera ammodytes meridionalis | E. Paros, Cyclades, Greece | M | AD | 225 |
| VA0095 | Vipera ammodytes meridionalis | S. Paros, Cyclades, Greece | M | AD | 348 |
| VA0104 | Vipera ammodytes meridionalis | W. Ano Koufonissi, Cyclades, Greece | F | AD | 246 |
| VA0110 | Vipera ammodytes meridionalis | W. Ano Koufonissi, Cyclades, Greece | M | AD | 273 |
| VA0136 | Vipera ammodytes meridionalis | NE. Antiparos, Cyclades, Greece | M | JUV | 174 |
| VA0141 | Vipera ammodytes meridionalis | NE. Antiparos, Cyclades, Greece | M | AD | 282 |
| VA0149 | Vipera ammodytes meridionalis | NE. Despotiko, Cyclades, Greece |  | JUV | 146 |
| VA0150 | Vipera ammodytes meridionalis | NE. Despotiko, Cyclades, Greece | M | AD | 276 |
| VA0154 | Vipera ammodytes meridionalis | Mt.Ossa, Thessalia, Greece | NaN | NaN | NaN |
| VA0155 | Vipera ammodytes montadoni | Macedonia/Thraki, Greece | NaN | NaN | NaN |
| VA0161 | Vipera ammodytes meridionalis | Mt. Tzoumerka, Epirus, Greece | NaN | NaN | NaN |
| VA0162 | Vipera ammodytes meridionalis | Mt. Oympos, Greece | NaN | NaN | NaN |
| VA0166 | Vipera ammodytes meridionalis | Prespa Lake, Greece | NaN | NaN | NaN |
| VA0171 | Vipera ammodytes meridionalis | Tebloni, Corfu, Ionian islands, Greece | NaN | NaN | NaN |
| VA0212 | Vipera ammodytes meridionalis | S. Mykonos, Cyclades, Greece | F | AD | 284 |
| VA0213 | Vipera ammodytes meridionalis | S. Mykonos, Cyclades, Greece | F | AD | 380 |
| VA0214 | Vipera ammodytes meridionalis | W. Mykonos, Cyclades, Greece | M | AD | 284 |
| VA0215 | Vipera ammodytes meridionalis | SE. Tinos, Cyclades, Greece | F | AD | 368 |
| VA0217 | Vipera ammodytes meridionalis | SE. Tinos, Cyclades, Greece | M | AD | 408 |
| VA0218 | Vipera ammodytes meridionalis | SE. Lefkada, Ionian islands, Greece | NaN | NaN | NaN |
| VA0219 | Vipera ammodytes meridionalis | SE. Ithaki, Ionian islands, Greece | NaN | NaN | NaN |
| VA0220 | Vipera ammodytes meridionalis | NW. Peloponnese, Greece | NaN | NaN | NaN |
| VA0222 | Vipera ammodytes meridionalis | Between Chora and Bouri, Ios, Greece | M | AD | 298 |
| VA0228 | Vipera ammodytes meridionalis | Syringas, Syros, Cyclades, Greece | M | AD | 295 |
| VA0311 | Vipera ammodytes | Adelfia island, Sporades, Greece | F | AD | 232 |
| VA0313 | Vipera ammodytes | Adelfia island, Sporades, Greece | M | AD | 359 |
| VG0188 | Vipera ursinii graeca | Tymfristos, Fthiotida, Greece |  |  |  |

Using the NGS method, ddRADseq, on our 39 samples (38 *Vipera ammodytes* and one *Vipera graeca*) allowed us to obtain on average 5992687 reads per individual after filtering. Samples that had a missing data percentage greater than 80% we also excluded from RAxML and STRUCTURE (Table 4.2).

Table 4.2: Number of Reads Retained and Percentage Missing Data per Sample.

| **Sample ID** | **# Reads Retained after filtering** | **% Missing data** |
|---|---:|---:|
| VA0001 | 6409407 | 5 |
| VA0002 | 6179358 | 13 |
| VA0005 | 495487 | 80 |
| VA0006 | 15383285 | 8 |
| VA0019 | 6896798 | 12 |
| VA0038 | 1459313 | 60 |
| VA0051 | 5625727 | 11 |
| VA0073 | 714827 | 63 |
| VA0076 | 15224680 | 17 |
| VA0077 | 3094109 | 28 |
| VA0090 | 2640511 | 16 |
| VA0091 | 8171414 | 14 |
| VA0093 | 4179870 | 27 |
| VA0095 | 4360546 | 23 |
| VA0104 | 2190234 | 40 |
| VA0110 | 6671408 | 4 |
| VA0136 | 4352438 | 7 |
| VA0141 | 14172048 | 10 |
| VA0149 | 8369229 | 8 |
| VA0150 | 12657706 | 9 |
| VA0154 | 1630053 | 42 |
| VA0155 | 5845855 | 28 |
| VA0161 | 2029657 | 45 |
| VA0162 | 1481097 | 46 |
| VA0166 | 6593435 | 15 |
| VA0171 | 1439185 | 51 |
| VA0212 | 1464724 | 49 |
| VA0213 | 6865740 | 8 |
| VA0214 | 3430878 | 37 |
| VA0215 | 9808671 | 6 |
| VA0217 | 9040534 | 22 |
| VA0218 | 4839264 | 23 |
| VA0219 | 5846544 | 24 |
| VA0220 | 2939795 | 24 |
| VA0222 | 17718913 | 9 |
| VA0228 | 12205360 | 20 |
| VA0311 | 3642147 | 24 |
| VA0313 | 5043124 | 17 |
| VG0188 | 2601423 |  |

## 4.2 RAxML

From the maximum-likelihood phylogenetic tree below and map we can see that there is a distinct difference between individuals found on the northern Cycladic islands (blue) versus those found on the southern Cycladic islands (green). The samples that were used from the mainland were also distinctly different from those clades found on the islands (Figure 4.1).

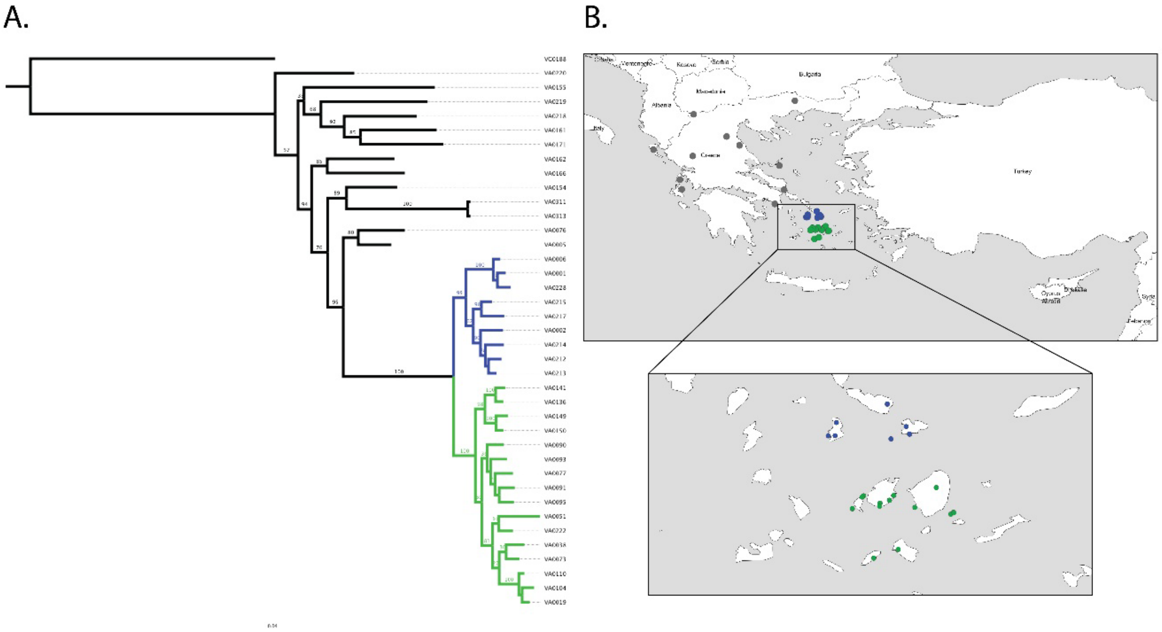

Figure 4.1: Maximum-likelihood phylogenetic tree and map showing the pattern of divergence of *Vipera ammodytes* in the Cyclades and Continental Greece.

## 4.3 STRUCTURE

STRUCTURE HARVESTER determined that a K (number of ancestral populations contributing to current genetic variation) of 6 was most highly supported, although we can see that there is a significant difference between 3 of the groups (Figure 2). The STRUCTURE plot below shows the same distinct pattern of diversification between *Vipera ammodytes* populations found on the northern islands (blue) versus those found on the southern islands (green). The two samples indicated in purple (VA0005 and VA0076) were obtained from populations found on the mainland. We can this see that the populations currently inhabiting the mainland are also very genetically distinct from the populations found on the islands. This STRUCTURE plot thus provides further support that the populations found on the northern and southern islands are genetically distinct (Figure 4.2).

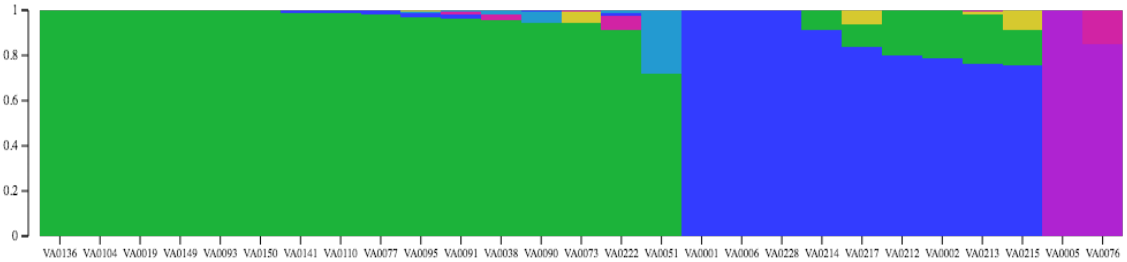

Figure 4.2: STRUCTURE plot showing the genetically distinct populations found on the northern and southern Cycladic islands as well as the mainland.

# CHAPTER 5

# DISCUSSION

Through looking at the RAxML tree and STRUCTURE plot we can see that there is a very distinct difference between the populations of *Vipera ammodytes* found on the northern islands versus the populations found on the southern islands. These figures also show that the mainland populations are significantly different from the populations found in the Cyclades.

Our results also supported and built on those from a previous study done on the divergence patterns of *Vipera ammodytes* in the Cyclades. In this study the author used four samples, two from the northern and two from the southern Cycladic islands, when he concluded that there may be a significant difference between the two (Ursenbacher *et al.* 2008). Through using 38 samples in our study we were able to obtain a clearer picture of this divergence pattern. Not only did we see that there is a distinct difference between the populations from the northern and southern Cycladic islands, but also between populations found within these islandic groups. Our results are also further supported by studies done on other species found on these islands. This same pattern of divergence between northern and southern island populations was witnessed in *Laudakia stellio*, *Podarcis milensis* and *Mesobuthus gibbosus* (Parmakelis *et al.* 2006).

In order to give an explanation for this divergence pattern we studied the geological history of the islands. We found that tectonic events lead to the separation of the Cyclades from continental Greece starting in the late Miocene (Anastasakis *et al.* 1990). The islands were then further broken up into the northern and southern Cycladic islands by the Zanclean flood in the early Pliocene (Roussos 2015). Additional fragmentation occurred during the upper Tertiary and Quaternary due to sea level changes caused by glacial and interglacial cycles that lead to the formation and disappearance of land bridges between the islands (Sagonas *et al.* 2014). The degree of connectivity between the islands also depended on the magnitude of the sea level change that occurred during these cycles (Roussos 2015).

The geological history of the islands are also well reflected within our results. In the STRUCTURE plot one can see a distinct difference between the populations of *Vipera ammodytes* found on the northern and southern islands, as well as on the mainland. This very distinct difference tells us that there was very limited gene flow between these islands. Given that *Vipera ammodytes* also does not appear to swim (Roussos 2015), the degree of connectivity with the mainland and within the norther and southern group had to be much less.

Due to the distinct differences between the northern and southern Cycladic groups, one could consider them to be evolutionary significant units (ESUs) which need to be considered in conservation efforts. A lot of different taxa also appears to have this same pattern of diversification as *V. ammodytes*, and if we were to lose one of the Cycladic groups, we would be losing a distinct pool of genetic variation. This loss would have a dire effect not only on all the species that show this pattern of diversification, but also on the ones that are yet to be studied. Conservationists need to look more closely into the genetic variations present on the islands. This would allow them to make more informed decisions regarding human development and expansion while still protecting the biodiversity.

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# BIOGRAPHICAL INFORMATION

Marelize was born and went to school in a small town in South Africa. After graduating from high school, she decided to attend the University of Texas at Arlington to pursue an Honors Bachelor of Science in Biology. Following her interests in genetics, Marelize joined Dr. Todd Castoe's genomics lab during the summer of 2015. One of the projects that she assisted with involved a species of water fleas, *Daphnia ambigua.* The goal of the project was to identify the mechanism underlying transgenerational plasticity. A second project that she is currently assisting with, and which will be used for her Honors Thesis, the lab hopes to identify how icelandic features and ecological shifts in the Cycladic archipelago, Greece, have influenced the evolution of different populations of nose-horned viper, *Vipera ammodytes.* During the Spring 2017 semester, Marelize also joined Dr. Sen Xu's Lab and will be continuing in his lab as a PhD student this coming Fall semester. Currently her project in the lab involves studying the reproductive cycles of Daphnia and the underlying mechanisms. The goal of this project is to determine what changes happen during meiosis, which allow cyclic parthenogenic Daphnia to switch reproductive methods. She aspires to become a scientist in the future.