MASTER IN BIOLOGY OF ORGANISMS AND ECOLOGY

Population genetics of Myosotella snails in the Azores

Ramos-Prieto, Rachel

Award date:
2024

Awarding institution:
University of Namur
Université Catholique de Louvain

Université Catholique de Louvain

École de biologie

Université de Namur

Département de Biologie

Population genetics of *Myosotella* snails in the Azores

Ramos-Prieto Rachel

Mémoire présenté en vue de l'obtention du diplôme
de Master en Biologie des Organismes et Ecologie

Promoteur : Alice Dennis

Année académique 2023-2024

ABSTRACT

Like many other archipelagos, the Azores are good naturel model to study speciation leading to numerous population genetics analysis particularly on plants. These islands are also rich in gastropods species. *Myosotella* snail are intertidal gastropods found almost everywhere in the world and that has been studied by de Frias Martins and Marques Mendes (2013) in the Azores. They have put in light possible species names based on penile complex morphology as well as a comparison with molecular analysis. The aim of this study is to go deeper inside molecular work by investigating the population genetics of *Myosotella* within the Azores. To achieve this, samples were taken from different populations of different islands and were amplified by PCR to be later sequenced. These sequences were used to build a phylogenetic tree and a haplotype network. Both the tree and the network show three distinct groups called A, B and C confirming the presence of genetic diversity inside the Azores. Some islands (or island's site) also have more than one haplotype which confirms that genetic diversity within island exist too. Unfortunately, linking genetic diversity with population's location wasn't possible for all sampling sites because of distant haplotypes. Many hypotheses arise from this results like human errors during laboratory experiments, the existence of different populations/groups/species inside a same sampling site or the possibility of sexual genetic differences (faster mutation rate inside male). This study wasn't optimised especially because the primer sets that worked randomly among samples but also because of lack of understanding about basic biology of *Myosotella*. Further work needs to be done taking these two issues into account to obtain the most reliable results and interpretations.

Keywords : *Myosotella*, Population genetics, Azores, Phylogenetic tree, Haplotype network, Islands, Gastropods

INTRODUCTION

The Azores archipelago

Isolated oceanic archipelagos are good places to study evolution in situ, including speciation. The Azores (**Figure 1**) are located between two continents : America and Europe (Ávila *et al.*, 2009; Schaefer *et al.*, 2011). By being ancient volcanic islands that arose from the sea, they were colonized by species from other regions. These islands are located near the Gulf stream current which originates in the Americas. But the biota is more similar to European species than American ones, raising questions about origins of species, colonization routes and speciation (Ávila *et al.*, 2009).

Most studies of species in archipelagos have found genetic variations between islands, and the Azores is no exception. Indeed, genetic diversity is high inside the Azores for the Azores lettuce *Lactuca watsoniana* (Dias *et al.*, 2016). One study, based on eight species of plants in the Azores, shows that different haplotypes exist among populations and sometimes haplotypes are specific of an island (Schaefer *et al.*, 2011). Genetic data may also help to understand the age of colonization of a species. Indeed, high genetic diversity may be the results of old colonization because of mutations accumulation but multiple colonization also conducts to genetic diversity, but in this case, the amount of shared alleles with the

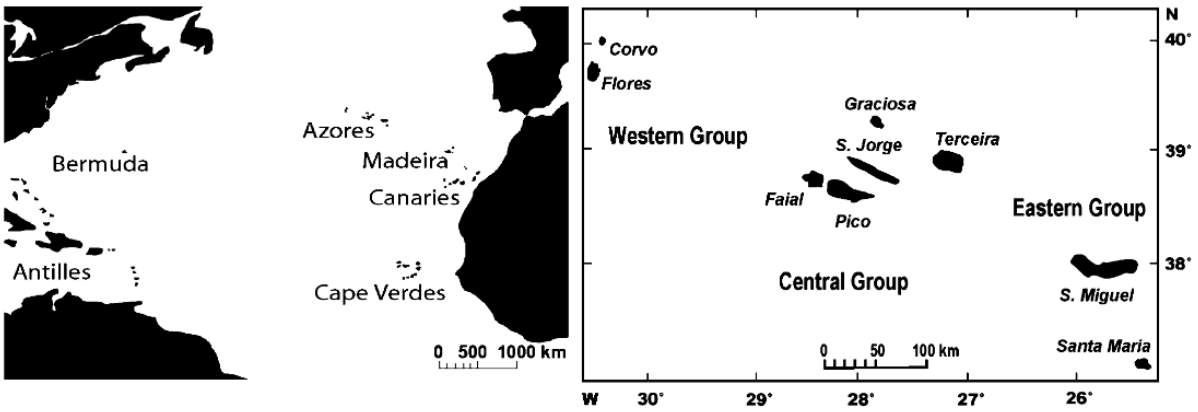


Figure 1 : Location of the Azores in the Atlantic Ocean and location of azorean islands inside the archipelago. Islands are separated in three groups : Western, Central and Eastern (Schaefer et al., 2011).

mainland is higher (Austerlitz *et al.*, 1997; Eales *et al.*, 2010; Rumeu *et al.*, 2011). On the other hand, recent colonization may results in lower genetic diversity because the population is isolated from the source population (Kaňuch *et al.*, 2014; Rumeu *et al.*, 2011).

Most examples based on plants have found high genetic diversity as well as different haplotypes inside the Azores and sometimes one haplotype per island (Dias *et al.*, 2016; Schaefer *et al.*, 2011). This may not represent all species, but they are a good start to investigate these topics in other taxa. Focusing on animals, the Azores seems to show a lack of genetic structure because of high gene flow (current or historical) at least in birds. But one population of *Regulus regulus*, the goldcrest, from Flores island is genetically different from other islands (Rodrigues *et al.*, 2014). The Azores also contained a high richness of molluscan species with 281 different species of Gastropoda, and littoral gastropods are part of the most studied species in the Azores (Cordeiro *et al.*, 2015). In general, the Macaronesian islands are good natural models to study speciation especially for intertidal species (Hawkins *et al.*, 2000).

Local adaptation

A successful mating leads to a movement of alleles between genetic pool. This phenomenon is called the gene flow and can be follow by the establishment of these alleles (Tigano and Friesen, 2016). Adaptations that specialize to a region or a location mostly occur if gene flow from other region is low, and if there is a low dispersion as well as active habitat choice. Gene flow tends to homogenize populations and disrupt local adaptations by bringing non adapted genotypes. If gene flow is low, there is no dilution effect of adaptation(s) because of external genotypes (Kawecki and Ebert, 2004; López-Goldar and Agrawal, 2021; Sanford and Kelly, 2011). This happen only if the selective pressure is not strong enough. Indeed, a certain amount of gene flow is still necessary to increase genetic diversity that can bring advantageous alleles and promote local adaptation (López-Goldar and Agrawal, 2021; Tigano and Friesen, 2016).

Gene flow is also influenced by the fine-grained persistent environmental variation, both biotic and abiotic, causing environmental heterogeneity (Sanford and Kelly, 2011). This kind of gradient is particularly present in nearshore habitats, caused by

oceanographic features leading to a spatial depending selection (Sanford and Kelly, 2011).

Genetic tools are of significant importance in understanding phylogeny and taxonomy, but they are also invaluable to measure phylogeography and population connectivity (or gene flow). This is in place of morphology, which can be difficult to analyze because of similarities, environmental plasticity, or even hybridization (DiBattista *et al.*, 2010; Le Roux and Wieczorek, 2009; Rijal *et al.*, 2015). High genetic diversity can be the result of multiple introduction which can lead to local adaptation or even population divergence (Le Roux and Wieczorek, 2009; Sheidai *et al.*, 2014). Multiple introductions can create new haplotypes by recombination (Le Roux and Wieczorek, 2009). Studying population connectivity and phylogeography using genetic methods can help to make links with geographical barrier or local adaptations for example (Rodrigues *et al.*, 2014).

The genus Myosotella

Myosotella, also called the "mouse-eared snail", is a hermaphroditic snail from the class Gastropoda and is found within the pulmonate order of Basommatophora and the family Ellobiidae, one of the most primitive families among pulmonates (Heller, 2015; Kh Al-Khafaji *et al.*, 2021; Marín, 2021; Mitov, 2016; Morton and Yonge, 1997; Robbins and Griffiths, 2023; Seelemann, 1968). Pulmonates are capable of breathing atmospheric oxygen outside water, and are found in tidal zones and estuaries, which are extreme environment for other marine species (Heller, 2015). Pulmonates are the most prolific molluscs in terms of invasion (Heller, 2015).

Myosotella are located in the intertidal zone with saline coastal environment, like salt marshes, estuaries, cobble and pebble beaches. They are mainly found under vegetation and rocks (De Frias Martins and Marques Mendes, 2013; Heller, 2015; Herbert, 2012; Kh Al-Khafaji *et al.*, 2021; Marín, 2021; Mitov, 2016; Robbins and Griffiths, 2023; Romero *et al.*, 2016). These snails live in the North Atlantic and Mediterranean regions but are known to be invasive in many other part of the world (Heller, 2015; Marín, 2021; Mitov, 2016). *Myosotella* are found all around the world except in the tropics and are most commonly found in European's coasts (De Frias Martins, 1996; Kh Al-Khafaji *et al.*, 2021). They likely originate in the eastern Atlantic and Mediterranean coasts as well as the Black sea, especially in the Northwestern area (De Frias Martins and Marques Mendes, 2013; Herbert, 2012; Linetskii *et al.*, 2020). But *Myosotella* may also be invasive in some parts of the world such as North, Central and South America, South Africa, Australia and New-Zealand (De Frias Martins, 1996; Herbert, 2012; Kh Al-Khafaji *et al.*, 2021).

Myosotella are not good dispersers and are stuck into the intertidal zone. In fact, going inland is impossible because *Myosotella*'s eggs don't tolerate fresh water (Hawkins *et al.*, 2000; Sander, 1967). Furthermore, going into water is also impossible for them because they don't tolerate long periods under water and moving on soft seabed is difficult for them (Sander, 1967). In addition, *Myosotella* larvae are not pelagic nor planktotrophic, which limits natural dispersion. Then, introduction may be made by eggs (or even adults) attached to an object (Blakeslee *et al.*, 2021; Gillespie, 2007; Herbert, 2012; Kh Al-Khafaji *et al.*, 2021).

A hypothesis for *Myosotella* dispersion is the attachment of individuals to other organisms like marine turtles, seaweed or even birds. Indeed, a common kind of dispersion used among intertidal molluscs found in the Azores is to be attached to birds' feathers and transported to new environments. This practice has been documented for terrestrial snails to disperse from Europe to the Azores thanks to intense winds leading birds. It is possible that intertidal snails may have been disperse using the same way. Another hypothesis is the attachment of snails to floating materials like logs or pumice (Ávila *et al.,* 2009; Blakeslee *et al.,* 2021).

Myosotella larvae are direct developer (Ávila *et al.,* 2009; Herbert, 2012). Most of the time, planktotrophic larval development (indirect development) species are found in higher number on islands than non-planktotrophic species (Ávila *et al.,* 2009). Direct developer has a higher connectivity between populations, and then a high gene flow, leading to a lower genetic differentiation between populations. Sometimes, larvae suffer from a higher mortality rate leading to a decrease in genetic variability. Despite all of that, direct development species still seems to have a large distributional range and may be successful colonizers, even better than pelagic larvae, because juveniles stay close from their parents after colonization helping the growth of the population (they didn't spread far apart). It correlates with the Allee effects : if the population density is to low, the population grow may slow or even decrease the size of the population (Blakeslee *et al.,* 2021; De Wolf *et al.,* 2000). This leads to another hypothesis explaining *Myosotella* dispersion.

Myosotella species have many different names around the world, mainly because of their high plasticity, especially of the shell, which can confuse identification (De Frias Martins and Marques Mendes, 2013; Herbert, 2012). Currently, many of these different names are gathered within two main species : *M. myosotis* and *M. denticulata*. *M. denticulata* can be distinguished from *M.myosotis* by its marine habitat (Anderson and Rowson, 2020).

Concerning Azorean species, internal penile complex anatomy has been used to show more fine-scaled diversity and to better understand taxonomy. Three different shapes were recognized before Azorean studies : flap (membranous), hooded (protected by a hood) and club (with two-pronged papilla). *Myosotella* possessing a flat shape are located in eastern islands of Santa Maria and São Miguel. *M. bicolor* are located in central islands and have a club shape. But an interesting point is the discovery of a new shape and two new species which possess it in western islands of Corvo and Flores : the twin shape (two papilla unprotected). The names of these two new species are *M. muchomacho* and *M. florentina* (De Frias Martins and Marques Mendes, 2013).

In addition, a molecular analysis was made to compare with penis anatomy. They choose to study ITS1 of rDNA. This analysis showed similar conclusion concerning the separation of populations from the Azores. But, one intriguing point is the link between flat shape Azorean populations and Portuguese hooded one (*M. myosotis*) (De Frias Martins and Marques Mendes, 2013).

Myosotella is an interesting genus to investigate population genetics in the Azores. Previous work in the archipelago has shown possible novel diversification, and representation of all types found on the mainland,

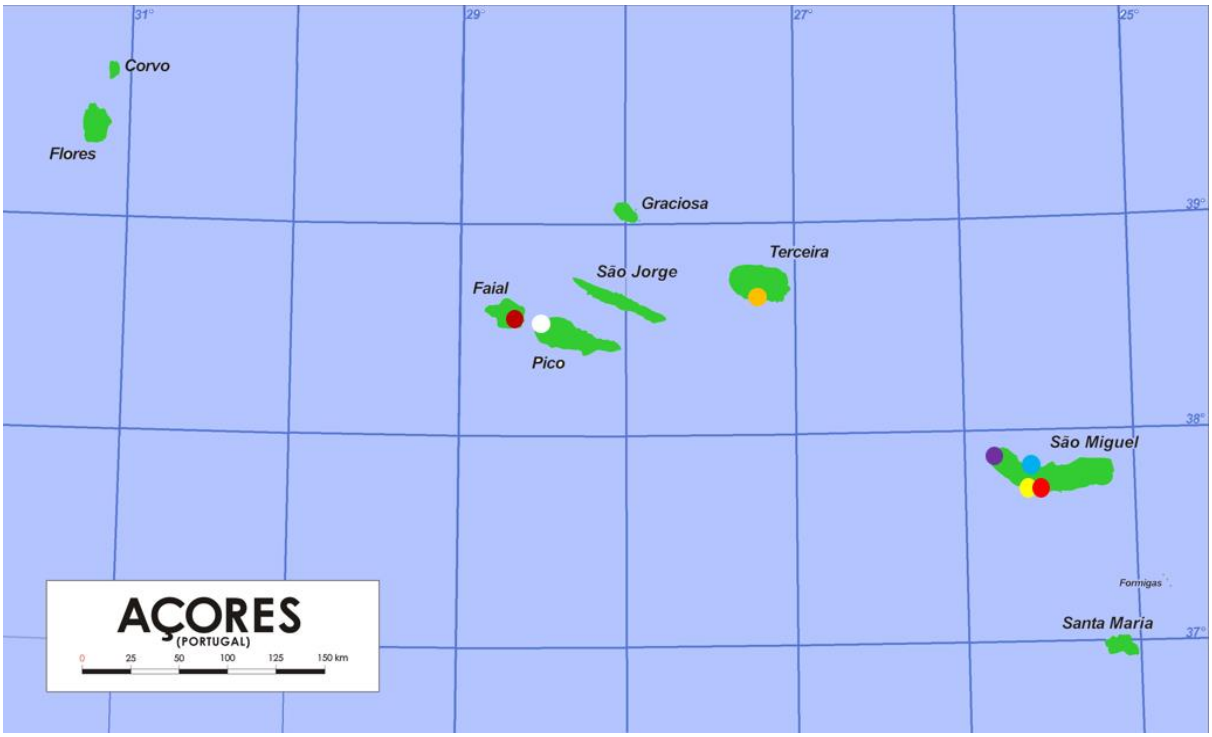


Figure 2 : Map of sampling location, each dot represents a site. Dark red is Horta, Faial. White is Areia Larga, Madalena, Pico. Orange is Ponta do Alcaide, Silveira, Terceira. Light red is site 1 Atalhada, w of Lagoa, Sao Miguel. Yellow is site 2 Brejela Atalhada, Sao Miguel. Purple is site 3 Ponta da Ferraria, Sao Miguel. Blue is site 4 Fenais da Luz, Sao Miguel.

but as of yet there are very few data yet. Pushing further in this topic could bring new understanding of this species or population genetics of gastropod in the Azores in general. I will test two alternative hypothesis here: (1) There are genetic differentiations between islands of the Azores as well as genetic variation inside populations (2) Genetic diversity is correlated to sites location potentially because of local adaptations and/or speciation. The two null hypothesis are (1) No genetic differences between islands and inside populations (2) There is no link between sites location and the genetic diversity.

MATERIALS AND METHODS

To analyse population genetics of *Myosotella* in the Azores, the mitochondrial gene CO1 (Cytochrome Oxidase 1) was chosen for sequencing. This gene is widely used as a barcode of species identification, because of its relatively high mutation rate, and has already been used to describe population structure in many gastropods, such as *Melarhaphe neritoides* (Fourdrilis *et al.*, 2018). But mitochondrial DNA is also only inherited by the maternal line (Ghiselli *et al.*, 2019), so this needs to be taken into account in its interpretation.

Sampling

All snail were collected by our master thesis supervisor Alice Dennis as well as António M. de Frias Martins between 3rd October 2012 and 9th May 2023. There is in total seven different sampling site spread overall several islands of the Azores but also four sites inside a same island (Sao Miguel) (**Figure 2**).

DNA extraction

DNA extraction was done on primarily foot tissue and sometimes parts of the shell if snails were too small. Whenever possible

Table 1 : Table representing all design primers sequences.

Primer name	Sequence
MyA405F	TGGTGCGTCGGTGGATTTAG
MyA118R	TGAAAGTGTGCCACCACGTA
MyA899F	TCACCGCTGCCACCATAATTAT
MyA1515R	CACGGTAGTCTCCAGGTTGC
MyB429F	CTTTTCGCTACACCTTGCTGG
MyB1123R	CGTAATGGAAGTGGGCTACCA
MyB933F	TGGAATTAAGGTGTTCAGGTGACT
MyB1519R	CTGTAACCGTCGTCTCCAGG

only a portion of the snail was used for the extraction, leaving tissue to extract this individual again in the future. The kit used for extraction was the DNeasy Blood and tissue kit from QIAGEN (cat no : 69504, Venlo, Netherlands) and it was used according to the protocol, except the digestion with proteinase K that was incubated overnight.

DNA quality was initially evaluated using a 2% agarose gel and a Nanodrop 2000 (by Thermo Fisher Scientific, Waltham, Massachusetts, US) was used to measure the concentration of DNA (Rijal *et al.*, 2015). Once protocols were established, only the Nanodrop was used to evaluate the DNA.

PCR and Sequencing

Successful extractions were next used for PCR (Polymerase Chain Reaction) to amplify a portion of CO1. First, the "universal" metazoan primers HCO1 and LCO1, from (Folmer *et al.*, 1994), were used but these failed to amplify CO1. As a results, we design four sets of primers based on two Myosotella's mitochondrial genome on GenBank (references : NC_012434.1. and JN606067.1). The first set was named MyA405F and MyA118R, the second set MyA899F and MyA1515R, the third set MyB428F and MyB1123R and the fourth set MyB944F and MyB1519R (**Table 1**). Many PCR conditions (like primer, temperature step, addition of different quantity of MgCl2 or even number of cycles) were tested before findings the best one for *Myosotella* populations (**Annexe 1**).

The final master mix (50µl for eight samples) was composed of 10µl of buffer 5X, 1µl of dNTP, 4µl of MyB429F primer, 4µl of MyB1123R primer, 0,24µl of GoTaq® polymerase (Promega), 26,76µl of water, 2µl of MgCl2 and 2µl of DNA. The PCR conditions were 2:30min at 94°C followed by 40 cycles of : 45 seconds at 94°C, 1 minute at 52°C, 1 minute at 72°C. Once these 40 cycles were finished, there was 10 minutes at 72°C and a decrease at 18°C when the PCR was finished. The number of samples per PCR varies between 7 and 23 and a 96 plate was used only once but not only with Azorean samples but also worldwide samples too.

When the PCR was completely done, an electrophoresis gel was used to visualize the product and verify that the PCR has worked. To do that, the gel needed to be made with SybrSafe, agarose and a buffer and

polymerise for approximatively 30min. Each PCR samples were mix with a green loading dye to fill the wells of the gel. The electrophoresis lasts for 35 min before putting under UV. The portion of CO1 gene amplified was around 700bp (base pairs) long, a purple ladder (Quick-Load®) of 100pb was used to verify if the PCR on this gene worked. If under UV a single band was visualized at 700bp, it suggests that correct CO1 fragment was amplified (**Annexe 2**).

All the samples possessing a band at 700pb were sent to Sanger sequencing to the company GENEWIZ (from Azenta life sciences) in Leipzig, Germany, or the company Eurofins in Ebersberg, Germany. But before being sent, the samples needed to be cleaned to remove excess primers and dNTPs using Macherey-Nagel™ NucleoSpin™ PCR and gel clean up kit using the protocol included in the kit. To edit, visualize and align sequences, the program Geneious Prime 2023.1.2 (Biomatters Inc., Auckland, New Zealand) was used. The results were evaluated based on the absorption curves (stocked as AB1 files). These contain the most plausible bases, but manual curation was needed to correct aberrant results, to check for contaminations, and overall to evaluate the results. If all the sequence is of poor quality, the sequencing needs to be done again. Problems mostly occurs because of contamination, non-specific primer binding, or background noises (Crossley *et al.,* 2020). After that, sequences from the same individuals (forward and reverse) were aligned to one another, and once again the sequence of the gene was corrected based on the agreement between the two. Once all individuals were aligned, the alignment between individuals showed SNP's (Single Nucleotide Polymorphism), a place of the genome where the nucleotide is different from other sequence. For all the alignments, pairwise alignment with Geneious alignment (global alignment with free and gaps) were used with a cost matrix of 65% of similarity.

Phylogenetic tree and haplotype network

Bayesian phylogenetic trees were built using MrBayes (Huelsenbeck and Ronquist, 2001), implemented via in Geneious. To verify which DNA substitution model best fit the DNA alignments, the software MEGA11 was useful and particularly because of its model function helping to statistically find the best one (**Annexe 3**). The resulting parameters were GTR substitution models, a gamma rate variation. Outgroups were chosen on Blast® (NCBI, Bethesda, US) as *Polygyra cereolus* (NC_032036.1) and *Brachypodella costata* (KT602792.1) two gastropods. MrBayes was run with a gamma category of 4 and a chain length of 1 100 000 and a 9,09% burn-in length. The tree was run four times to verify the possibility of any variations.

The relationships among haplotype as well as the genetic distance (Chaisiri *et al.,* 2021) were visualized using a haplotype network on R and R studio with the help of different packages : "pegas" (Paradis *et al.,* 2023), "plyr" (Wickham, 2023) and "reshape" (Wickham, 2022). In fact, this network may put in light the genetic variations, distance and links between *Myosotella* populations around the Azores but also genetic variations inside a same population. To complete our data, previous sequences, made by other scientists, of *Myosotella* available on Blast® were used. This sequences are the same as used to design primers sets (references : NC_012434.1. and JN606067.1).

RESULTS

Before using MyB429F and MyB1123R primers. In total, 38 PCR were done with different conditions and concentrations because of the lack of bands on the electrophoresis gel and each samples have been amplified more than once (**Annexe 4**). Once we had enough samples that worked, they were sent to sequencing even if the success rate of band appearing was very low. Theses samples were sent three times to sequencing but the results were never of good quality with a lot of background noises. After analysing the best ones in Blast, it has been shown that many of them weren't even part of *Myosotella* genus. For example, some sequences were *Vibrio*, a bacteria.

Then, the primers were changed to MyB429F and MyB1123R. Nine PCRs were performed but once again the success rate of band appearing was low although still higher than with previous primers. Some samples were amplified several times with no results, and some worked after many attempts. The success appeared to be random. On the 53 samples, only 21 had sufficient sequence of suitable quality to be analysed in the phylogenetic tree and haplotype network.

Phylogenetic tree

The phylogeny resulting (**Figure 3**), shows that all *Myosotella* samples are different from the outgroups. There are also three distinct groups inside them represented by the letters A, B and C. These three groups are supported by branch values varying

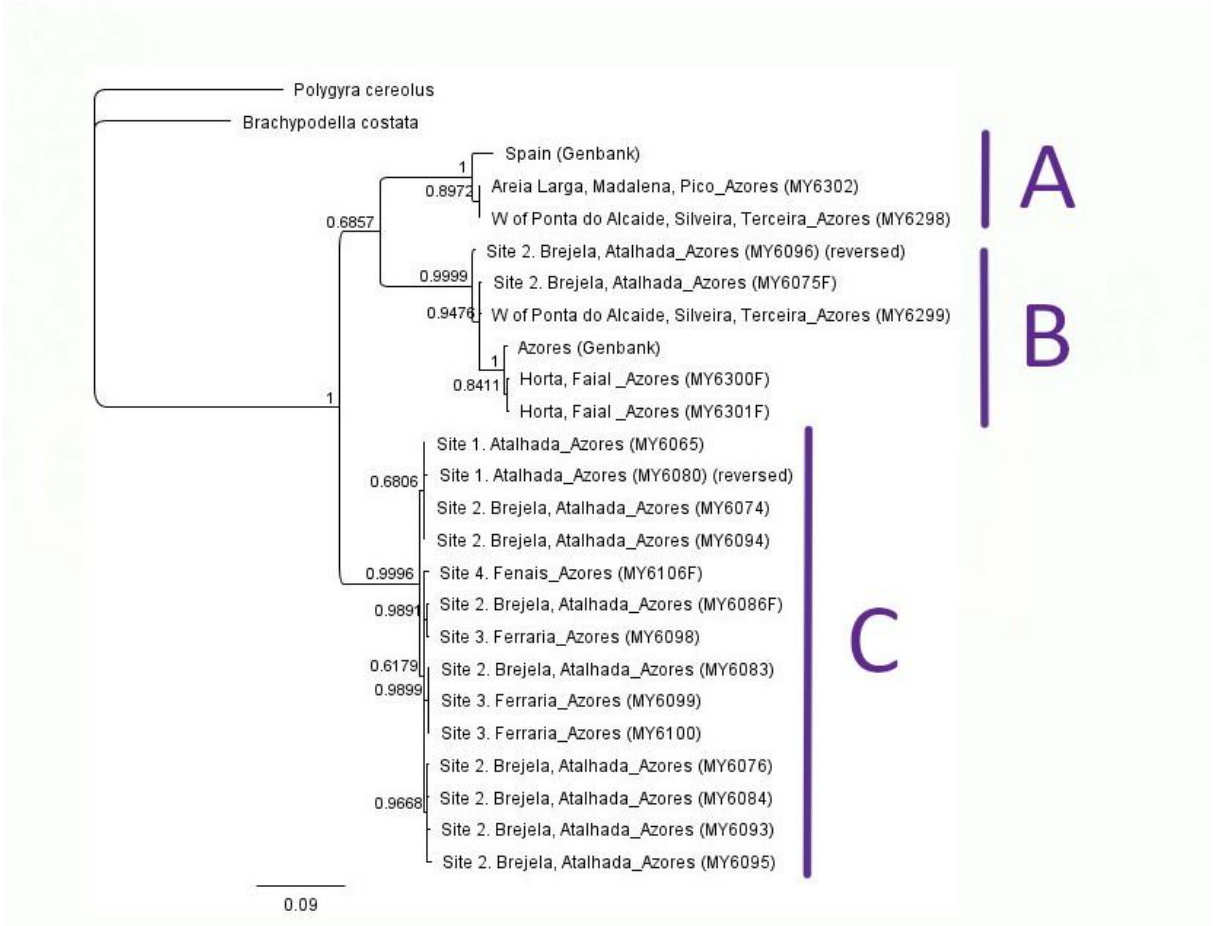


Figure 3 Phylogeny of Myosotella samples as well as the two-genome used to design primers (Spain and Azores Genbank). Polygyra cereolus and Brachypodella costata are two species of gastropods used as outgroups, their sequences were found in Genbank. A, B and C are the three distinct groups.

between 0.9996 and 1. The two genomes from GenBank used to design primers are also included inside the samples. The Spanish genome is found inside an Azorean groups (group A) while the Azorean genome is found near Faial Island samples (group B). Samples are not grouped by islands.

Site 1 is found only in group C. Site 2 is found in groups B and C. Site 3 is only group C, but one sample is separated from the two others and is with sample from site 2. Site 4 has only one sample found in group C and is a bit isolated. Both Faial Island's samples share the same node in group B. Terceira island's samples are part of group A and B. The only Pico island's sample is close to one Terceira's sample and not far away from the Spanish genome in group A.

Haplotype network

The haplotype network was made (**Figure 4**) with sequences that are 550 base pair long (samples needed to be cut to the same length to make the network). Each samples are links to one haplotype number (**Annexe 5**). It shows three clear distinct groups that are the same than previous ones and which will also be called A, B and C.

The group B is in between the two others group and is separated from them by many mutations. Inside group B and C, there is linear mutations unlike group A. Indeed, group A seems to contain a radiations of mutations around the haplotype 7 (Site 2,

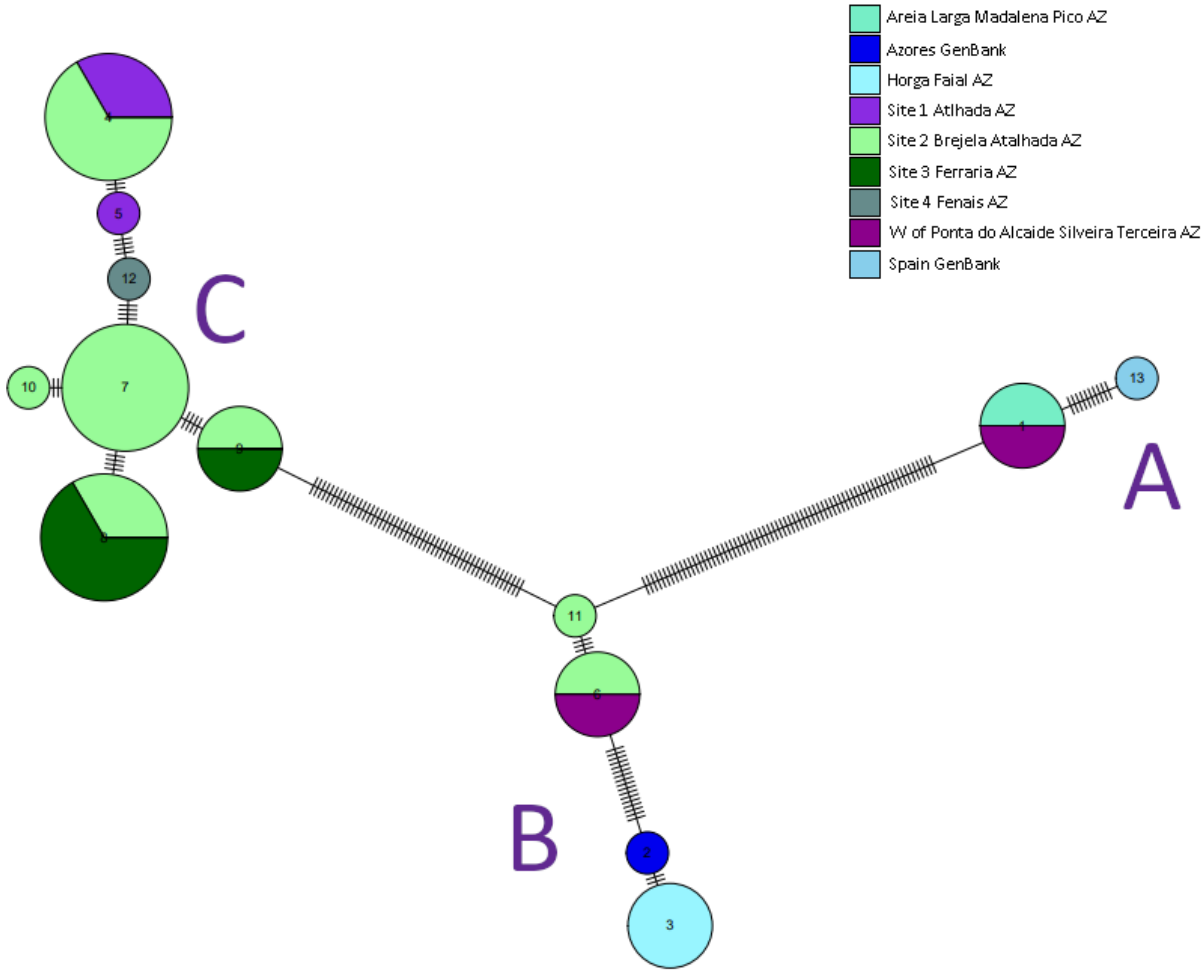


Figure 4 : Haplotype network of Azorean samples using R and R Studio. Each colour represents a location in the Azores, but also the two genomes took in GenBank referred to as Spain and Azores. Every bar represented on the network between haplotypes are equal to one mutation. Groups A, B and C are indicated.

Brejela Atalhada). One mutation branch goes to group B passing by haplotype 9 containing site 2 (My6086) and site 3 (My6098) samples. Another one creates the haplotype 8 with site 2 (My6083) and site 3 (My6099 and My6100) samples as well. Haplotype 10 also radiates from this and only includes a site 2 sample (My6095). The longest branch radiates at first by haplotype 12 (My6106, site 4 Fenais), then by haplotype 5 (My6080, site 1 Atalhada) and ending with haplotype 4 grouping site 1 (My6065) and site 2 (My6074 and My6094).

Group B is found in the centre of the network with the haplotype 11 (My6096). After some mutations, haplotype 6 appears with site 2 (My6075) and Terceira Island (My6299). After, many mutations separate haplotypes 6 and 2. Haplotype 2 only contains the Azorean genome from GenBank is genetically close, only few mutations, from haplotype 3 with Faial Island (My6300 and My6301)

Group C has two haplotypes. The first one, haplotype 1, is separated from the haplotype 11 of group B by a high number of mutations. Haplotype 1 is composed of Pico Island (My6302) and Terceira Island (My6298). In the end of the group C, splits by few mutations from haplotype 1, haplotype 13 is only defined by the Spanish genome from GenBank.

Due to the size of the sequence (only 354 base pair), Porto Novo, Vimeiro, Portugal (MY6290) has been deleted from the phylogenetic tree as well as the haplotype network. Such short sequence leads to a loss of information. The tree and the network including this samples can be found in annexes (**Annexes 6 and 7**).

DISCUSSION

To analyse population genetics of *Myosotella* snails in the Azores, the phylogenetic tree as well as the haplotype network of seven sites of the Azores and two mitochondrial genomes from GenBank are giving us a lot of information.

Samples in groups A, B and C of the tree and network correspond exactly to each other. It is clearly evident that group B is the link between group A and C in the haplotype network but not so much inside the tree. Indeed, the phylogeny groups A and B together, while and C is sister to the combined group of A and B.

By observing the distribution of sites haplotypes, one of the first things that stands out is the position of site 2 haplotypes. Indeed, inside site 2 Brejela Atalhada, haplotypes are extended between group C and B. But these two groups are separated by many mutations maybe because of isolation or reproductive barriers between them. But these population live on the same island and the same location on this island.

Then, the factor influencing such differences should be more local. And in fact, during snails sampling, it was noticed that some individuals were found under grass and other under rock (**Annexe 8**). Maybe, this leads to different adaptations depending on the nature of the soil. Indeed, habitat seems to have a role to play in juvenile dispersal and then to population structure for *Cingula trifasciata*, a microgastropod, within the Azores (Baptista *et al.*, 2021). But it is not the right explanation here, or at least not entirely. At first, if we look at group B, the two samples from site 2, My6075 and My6096, are living in a rocky habitat and can corroborate this hypothesis.

But if we look at group C, and especially at haplotype 7, the three samples from site 2 are not from the same kind of environment : two of them come from rocky habitat (My6076 and My6093) and one of them (My6084) come from a grassy habitat. In general, group C is composed of grassy and rocky samples which contradicts the hypothesis.

Sites 1, 2, 3 and 4 are different locations on the same island (**Figure 2**), the island of Sao Miguel. Site 1 Atalhada samples were found in front of the Observatorio Volcanologico e Geotermico, site 2, as said before, in Brejela Atalhada under rocks and grass, site 3 were found near hot baths in Ponta da Ferraria and site 4 were found in Fenais da Luz. They are all inside group C except some site 2 samples : MY6096 and My6075. Did they migrate to Sao Miguel from site 2 or did they find their origin in Sao Miguel and only then migrates to other islands trough site 2 ? In addition, group C only contain these Sao Miguel samples that are linked together with short branch length. Suggestions about that are (1) this group was more successful in the PCR and sequencing leading to higher number of samples of close relatives in the tree (2) this group has recent radiations, seen by the short branch length, and even a possible asexual reproduction inside this population.

Concerning Faial Island population, the only haplotype present is close to the Azorean genome found on Genbank. This genome is separated by two mutations from Faial meaning that they are two very close haplotypes. The exact location of the snail used for the genome is unknown, but this suggests that it could have come from Faial. In addition, we can see in **Figure 2** that Pico Island and Faial Island are in close proximity, but their haplotypes are far apart inside the network. In can mean that despite the close distance, gene exchange between these two populations may be low possibly because of an environmental barrier or human activities.

Terceira Island samples are split across both groups A and B. They are then differentiated by many mutational steps which is clearly strange because they are both coming from the exact same site. This may be explained by possible errors during samples sorting or during lab experiments : one of them may have been mistaken and is a Pico's sample or a site 2's sample. Even more because of the haplotype 11 right in between of Terceira's haplotypes (1 and 6). Alternatively, this location may contain two very different groups/populations/species mixed together.

António M. de Frias Martins and Ana Rita Marques Mendes proposed different species names of *Myosotella* based on different penis shapes found in Azorean islands. Based on their work and the locations of our snails, it seems that samples from Sao Miguel (Site 1 to 4) are *Myosotella myosotis* ("flat" shape). On the other hand, samples from Pico, Terceira and Faial should be *Myosotella bicolor* ("club" shape) (De Frias Martins and Marques Mendes, 2013). In addition, some localized populations of *M.myosotis* were found in Terceira and Faial which should normally be *M.bicolor* (De Frias Martins and Marques Mendes, 2013). Guessing the name of the species only based on the location is not the most reliable technique. Looking at the haplotype network and phylogenetic tree, it seems that all Sao Miguel samples are close and may be one species, possibly *M.myosotis*. But two samples are found closer to possibly *M.bicolor* samples. These two samples, MY6096 and My6075, can be localized

population of *M.bicolor* in Sao Miguel Island. But these hypothesis cannot be confirmed with certitude because of the lack of anatomical studies and electron microscopy in on our samples.

Another point is the various number of problems that occurred during experiments. First, finding the best primers set was a long process and even so these primers are not optimal. Newly designed primers were built based on two mitochondrial genomes from GenBank, but the set used in the end is matched to the Azorean genome, but the exact location is unknown. Exact name of species of the two genomes is also unknown. Primer success then appeared to happen randomly on the samples. In other word, there was not a primer set that matched/worked by supposed species. Maybe diversity is far more complicated than we see here, and many more species exist in the Azores but didn't work in the PCR step because the affinity with primers is too low. To improve this, we should have sequenced whole genomes of *Myosotella* sample for at least each location. Then building one set of primers for each location with a supposed species based on de Frias Martins and Marques Mendes (2013). With this approach, the PCR should have been more optimized but because of lack of time, it couldn't be done. Second, some samples that worked in the PCR (a clear band was visualised in the electrophoresis gel) didn't work even when the company tried many times. The primers were maybe not specific enough creating a lot of background noises. The extraction may also have a role to play, with poor quality DNA extraction (not enough DNA, too old, not enough ethanol concentration, etc.).

But the primer match is not the only explanations of the difficulties encountered with theses snail. It is known that by sequencing mitochondrial genes, nuclear mitochondrial pseudogenes (NUMTs) can also be sequenced by accident. These NUMTs show a slower rate of nucleotide substitution than their mitochondrial ancestor. NUMTs are well known to cause problems by being mistaken for mitochondrial DNA leading to errors when building phylogenetic tree as well as relationships between samples (Schultz and Hebert, 2022). NUMTs are particularly problematic if the PCR is used to amplify only one gene (Ghiselli *et al.,* 2021).

Contamination could also have been an issue during lab experiments as well as other human errors, as previously mentioned, like samples sorting mistakes or exchange between samples. Some samples relationships are strange so it's legitimate to ask questions about it. For examples, sites 2 split between group B and C and Terceira samples split between groups A and B are not very coherent, even if some hypothesis may exist.

Molluscan mitochondrial genomes also have a large amount of variability inside their structures and functions. This can be changes in size of the genome, the orientations of transcription, extensive gene order rearrangement as well as variation of architecture of genes (duplication, insertion and deletion), male specific genetic insertion, etc. (Ghiselli *et al.,* 2021). All of these phenomenon can result to strong difficulties to investigate phylogeny of mollusc and then by extension of *Myosotella.* Moreover, we don't know the sex of our snails, some genetic relationships among samples may be made by sex similarity more than location similarity. Indeed, males possess in general more cell division than female leading to a higher mutation rate (Hedrick, 2007). To

cite the example site 2, environment couldn't explain why two samples were found in group B and the rest in group C. Maybe these represent one snail of each sex at the same site, combined with some sex-specific mitochondrial inheritance. Terceira Island samples may also be both separated because of this sex genetic variations. Some examples of this sexual difference have been reported in the literature. The horse mussel, *Modiolus modiolus*, as well as the smooth-shelled blue mussel, *Mytilus edulis*, has longer branch length with male inherited mitochondrial genome meaning that males are evolving more rapidly (bivalves mitochondrial genomes can be inherited by both parents) (Robicheau *et al.,* 2017; Śmietanka *et al.,* 2013). But we are not sure about *Myosotella* reproductive system by now, snail can be hermaphrodite, gonochoric or even both of them depending on the population and the environment. Further works about it may be an interesting start to better understand genetic relationships.

CONCLUSION

Population genetics of *Myosotella* snails is not as easy as expected and may need deeper understanding. Our first hypothesis, stating that populations of *Myosotella* in the Azores shows genetic diversity between islands and inside island's population can be accepted. In fact, the phylogenetic tree as well as the haplotype network clearly put in light distinct genetic groups. Concerning diversity inside island's populations, we have many location that posses more than one haplotypes meaning that their population shows enough genetic diversity to distinguish genetic groups within them.

For the second hypothesis, we had stated that genetic diversity could be linked to sites locations because of possible local adaptations and/or speciation. Contrary to our hypothesis, genetic relationships between snail are not completely correlated with the location of the site, but it doesn't mean that some adaptation or speciation didn't occur. Maybe different sub-population, species or genetic group are living on a same island leading to distant relationships between snails of a same location. But these results need to be treated with caution because of all the primers problems, a lot of samples and sites locations never worked to the sequencing leading to an unequal number of samples per site. If we follow the work of de Frias Martins and Marques Mendes (2013), we must have two species of *Myosotella* inside our samples : *M.myosotis* and *M.bicolor*.

These results may be an opening to further work concerning *Myosotella* inside the Azores. There are still many things to investigate. The most important point is finding the best(s) primers set(s) to sequence all the Azorean populations and put in light to most realistic genetic relationship between populations and not just random samples sequencing. This can be done by sequencing whole genome of different populations increasing the chance to build the right set of primers and maybe on set per assumed species. To improve interpretation of results, analysing gene flow and possible barriers to gene flow is the most relevant thing to do. Concerning species, morphological studies must continue alongside genetic one to improve the knowledge about *Myosotella* phylogeny. The last point to improve is the understanding of *Myosotella* biology and anatomy, to distinguish male and female for the genetic analysis for example, but particularly what is their type of reproduction and how does it work ? This work has helped pave the way in our understanding of

Myosotella, but much remains to be discovered in the future.

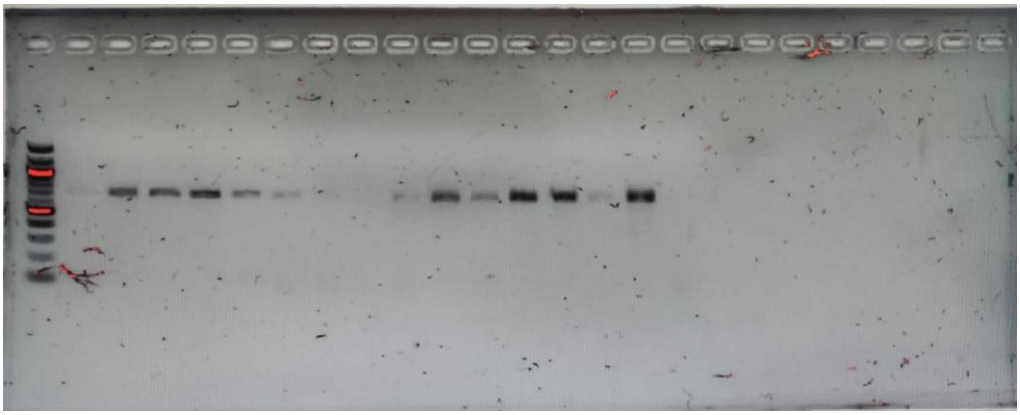
ACKNOWLEDGMENTS

I would like to thank Alice Dennis who supervised this master thesis and collected some of the samples used. I would also like to thank Ant6nio M. de Frias Martins who also help to collect new samples and gave us some of his old samples. Finally, I would like to thank Laura Cherpion who helped me during laboratory experiments.

ANNEXES

Annexe 1 : Table representing the number of PCR done for each primers tested to amplify one section of the gene CO1.

Primers	Number of PCR
HCO1 and LCO1	20
MCO1 and HCO1	1
16LRN 13398 and 16SRHTB	1
18SR174 and 18SF466	1
MyA420F and MyA118R	2
MyA899F and MyA1515R	4
MyB429F and MyB1123R	9
MyB933F and MyB1519R	1



Annexe 2 : Example of a post PCR gel that we have made in the lab. The first well is the ladder and after there is one sample per well. All the black lines at 700 bp are considered as a successful PCR of CO1 gene.

Annexe 3 : Table of statistics use on MEGA11 to find the fittest model for our sequences. The first one in the table is the fittest model to make the phylogenetic tree for our samples.

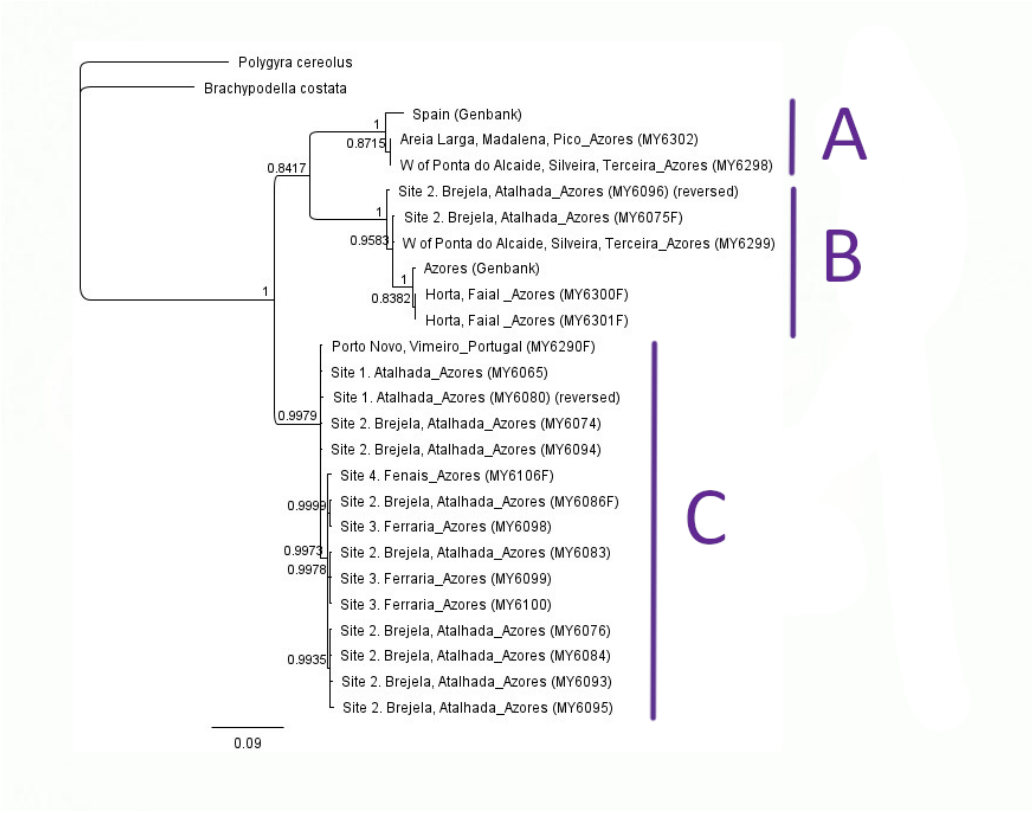
Model	#Param	BIC	AICc	lnL	Invariant	Gamma
HKY+I	54	5779,469463	5365,283831	-2628,455029	0,562529209	n/a
HKY+G	54	5780,494534	5366,308902	-2628,967565	n/a	0,264940686
HKY+G+I	55	5787,460795	5365,612006	-2627,612183	0,445912683	0,957452519
TN93+G	55	5790,128212	5368,279423	-2628,945891	n/a	0,263271856
T92+G	52	5793,629255	5394,770701	-2645,211952	n/a	0,261514815
TN93+I	55	5796,745904	5374,897114	-2632,254737	0,559488132	n/a
TN93+G+	56	5797,118266	5367,606572	-2627,602405	0,444701667	0,954760406
T92+I	52	5799,2016	5400,343045	-2647,998124	0,564216761	n/a
GTR+G	58	5802,737197	5357,900454	-2620,734844	n/a	0,275469379
T92+G+I	53	5804,727865	5398,205645	-2645,922743	0,421273091	0,838914602
GTR+I	58	5806,057601	5361,220858	-2622,395046	0,562297829	n/a
GTR+G+I	59	5819,253534	5366,754648	-2624,1545	0,459956601	1,148434326
K2+I	51	5893,43972	5502,245084	-2699,955697	0,56200375	n/a
K2+G	51	5898,123781	5506,929145	-2702,297727	n/a	0,28799746
K2+G+I	52	5905,38564	5506,527086	-2701,090144	0,372637174	0,727959262
HKY	53	5976,195684	5569,673464	-2731,656653	n/a	n/a
T92	51	5989,357571	5598,162935	-2747,914622	n/a	n/a
GTR	57	5997,088172	5559,913826	-2722,748845	n/a	n/a
TN93	54	5997,754964	5583,569333	-2737,59778	n/a	n/a
JC+G	50	6009,461806	5625,931341	-2762,805253	n/a	0,345348282
JC+I	50	6011,351702	5627,821237	-2763,750201	0,546616957	n/a
JC+G+I	51	6032,154727	5640,96009	-2769,3132	0,453915473	1,761181983
K2	50	6073,319951	5689,789486	-2794,734325	n/a	n/a
JC	49	6176,150942	5800,284902	-2850,988334	n/a	n/a

Annexe 4 : Table representing the number of PCR done for each samples associated with to their location.

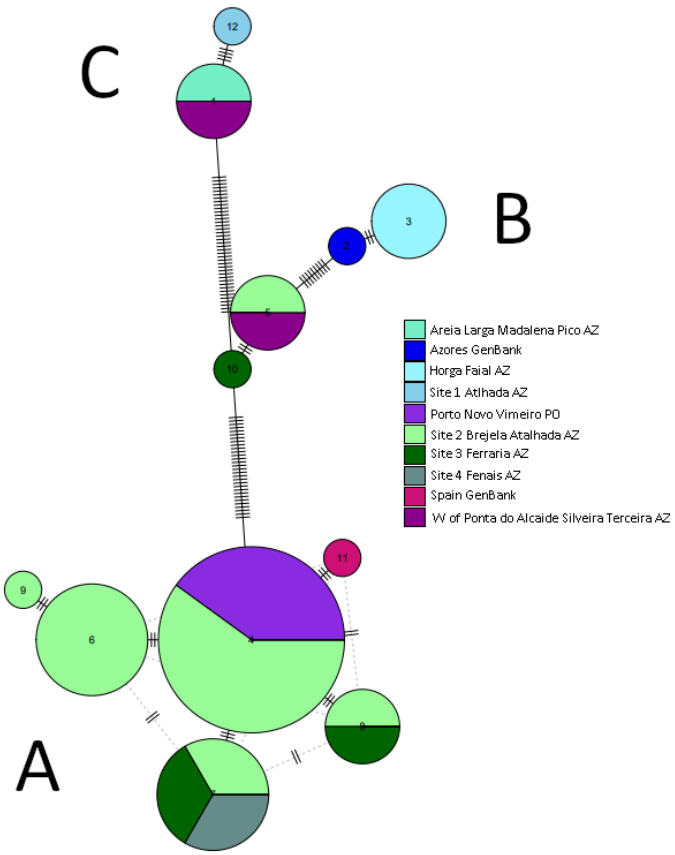
Samples	Location	Number of PCR done
My6065	Site 1. Atalhada	7
My6074	Site 2. Brejela, Atalhada	2
My6075	Site 2. Brejela, Atalhada	10
My6076	Site 2. Brejela, Atalhada	12
My6080	Site 1. Atalhada	1
My6083	Site 2. Brejela, Atalhada	3
My6084	Site 2. Brejela, Atalhada	5
My6086	Site 2. Brejela, Atalhada	13
My6093	Site 2. Brejela, Atalhada	18
My6094	Site 2. Brejela, Atalhada	4
My6095	Site 2. Brejela, Atalhada	13
My6096	Site 2. Brejela, Atalhada	13
My6098	Site 3. Ferraria	2
My6099	Site 3. Ferraria	1
My6100	Site 3. Ferraria	2
My6106	Site 4. Fenais	2
My6290	Porto Novo, Vimeiro	2
My6298	W of Ponta do Alcaide, Silveira, Terceira	2
My6299	W of Ponta do Alcaide, Silveira, Terceira	4
My6300	Horga, Faial	2
My6301	Horga, Faial	2
My6302	Areia Larga, Madalena, Pico	2

Annexe 5 : Table representing the haplotypes number given for each samples in the haplotype network. This data has been made using R and R Studio.

Samples	Sites	Haplotype
My6302	Areia Larga Madalena Pico AZ	1
GenBank	Azores	2
My6300	Horga Faial AZ	3
My6301	Horga Faial AZ	3
My6065	Site 1 Atalhada AZ	4
My6080	Site 1 Atalhada AZ	5
My6074	Site 2 Brejela Atalhada AZ	4
My6075	Site 2 Brejela Atalhada AZ	6
My6076	Site 2 Brejela Atalhada AZ	7
My6083	Site 2 Brejela Atalhada AZ	8
My6084	Site 2 Brejela Atalhada AZ	7
My6086	Site 2 Brejela Atalhada AZ	9
My6093	Site 2 Brejela Atalhada AZ	7
My6094	Site 2 Brejela Atalhada AZ	4
My6095	Site 2 Brejela Atalhada AZ	10
My6096	Site 2 Brejela Atalhada AZ	11
My6098	Site 3 Ferraria AZ	9
My6099	Site 3 Ferraria AZ	8
My6100	Site 3 Ferraria AZ	8
My6106	Site 4 Fenais AZ	12
GenBank	Spain	13
My6298	W of Ponta do Alcaide Silveira Terceira AZ	1
My6299	W of Ponta do Alcaide Silveira Terceira AZ	6



Annexe 6 : Phylogenetic tree that include Porto Novo, Vimeiro, Portugal sample. The three distinct group (A, B and C) remains the same.



Annexe 7 : Haplotype network that include Porto Novo, Vimeiro, Portugal sample. The three distinct groups (A, B and C) are a bit different.

Annexe 8 : Table representing environment of samples found in sites 2, Brejela Atalhada.

Samples	Environment
MY6074	Myosotella from very top of beach, at base of rock cliff
MY6075	Myosotella from very top of beach, at base of rock cliff
MY6076	Myosotella from very top of beach, at base of rock cliff
MY6083	Myosotella from grassy area
MY6084	Myosotella from grassy area
MY6086	Myosotella from grassy area
MY6093	Myosotella from rocks
MY6094	Myosotella from rocks
MY6095	Myosotella from rocks
MY6096	Myosotella from rocks

BIBLIOGRAPHY

Anderson, R., Rowson, B., 2020. ANNOTATED LIST OF THE NON-MARINE MOLLUSCA OF BRITAIN AND IRELAND, 2020.

Austerlitz, F., Jung-Muller, B., Godelle, B., Gouyon, P.-H., 1997. Evolution of Coalescence Times, Genetic Diversity and Structure during Colonization. Theoretical Population Biology 51, 148–164. https://doi.org/10.1006/tpbi.1997.1302

Ávila, S.P., Marques Da Silva, C., Schiebel, R., Cecca, F., Backeljau, T., De Frias Martins, A.M., 2009. How did they get here? The biogeography of the marine molluscs of the Azores. Bulletin de la Société Géologique de France 180, 295–307. https://doi.org/10.2113/gssgfbull.180.4.295

Baptista, L., Meimberg, H., Ávila, S.P., Santos, A.M., Curto, M., 2021. Dispersal ability, habitat characteristics, and sea-surface circulation shape population structure of Cingula trifasciata (Gastropoda: Rissoidae) in the remote Azores Archipelago. BMC Ecol Evo 21, 128. https://doi.org/10.1186/s12862-021-01862-1

Blakeslee, A.M.H., Miller, A.W., Ruiz, G.M., Johannesson, K., André, C., Panova, M., 2021. Population structure and phylogeography of two North Atlantic Littorina species with contrasting larval development. Mar Biol 168, 117. https://doi.org/10.1007/s00227-021-03918-8

Chaisiri, C., Liu, X., Lin, Y., Fu, Y., Zhu, F., Luo, C., 2021. Phylogenetic and Haplotype Network Analyses of Diaporthe eres Species in China Based on Sequences of Multiple Loci. Biology 10, 179. https://doi.org/10.3390/biology10030179

Cordeiro, R., Borges, J., Martins, A., Ávila, S., 2015. Checklist of the littoral gastropods (Mollusca: Gastropoda) from the Archipelago of the Azores (NE Atlantic). Biodiversity 6, 855–900.

Crossley, B.M., Bai, J., Glaser, A., Maes, R., Porter, E., Killian, M.L., Clement, T., Toohey-Kurth, K., 2020. Guidelines for Sanger sequencing and molecular assay monitoring. J Vet Diagn Invest 32, 767–775. https://doi.org/10.1177/1040638720905833

De Frias Martins, A., 1996. Anatomy and systematics of the western Atlantic Ellobiidae (Gastropoda: Pulmonata). Malacologia 37, 163–332.

De Frias Martins, A.M., Marques Mendes, A.R., 2013. Do cosmopolitans speciate speciate? Anatomical diversity of Myosotella in Azores. https://doi.org/10.13140/2.1.3483.4564

De Wolf, H., Verhagen, R., Backeljau, T., 2000. Large scale population structure and gene flow in the planktonic developing periwinkle, Littorina striata, in Macaronesia (Mollusca: Gastropoda). Journal of Experimental Marine Biology and Ecology 246, 69–83. https://doi.org/10.1016/S0022-0981(99)00177-X

Dias, E.F., Moura, M., Schaefer, H., Silva, L., 2016. Geographical distance and barriers explain population genetic patterns in an endangered island perennial. AoB PLANTS 8, plw072. https://doi.org/10.1093/aobpla/plw072

DiBattista, J.D., Wilcox, C., Craig, M.T., Rocha, L.A., Bowen, B.W., 2010. Phylogeography of the Pacific Blueline Surgeonfish, *Acanthurus nigroris*, Reveals High Genetic Connectivity and a Cryptic Endemic Species in the Hawaiian Archipelago. Journal of Marine Sciences 2011, e839134. https://doi.org/10.1155/2011/839134

Eales, J., Thorpe, R.S., Malhotra, A., 2010. Colonization history and genetic diversity: adaptive potential in early stage invasions. Molecular Ecology 19, 2858–2869. https://doi.org/10.1111/j.1365-294X.2010.04710.x

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers

for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3, 294–299.

Fourdrilis, S., de Frias Martins, A.M., Backeljau, T., 2018. Relation between mitochondrial DNA hyperdiversity, mutation rate and mitochondrial genome evolution in Melarhaphe neritoides (Gastropoda: Littorinidae) and other Caenogastropoda. Sci Rep 8, 17964. https://doi.org/10.1038/s41598-018-36428-7

Ghiselli, F., Gomes-Dos-Santos, A., Adema, C.M., Lopes-Lima, M., Sharbrough, J., Boore, J.L., 2021. Molluscan mitochondrial genomes break the rules. Philos Trans R Soc Lond B Biol Sci 376, 20200159. https://doi.org/10.1098/rstb.2020.0159

Ghiselli, F., Maurizii, M.G., Reunov, A., Ariño-Bassols, H., Cifaldi, C., Pecci, A., Alexandrova, Y., Bettini, S., Passamonti, M., Franceschini, V., Milani, L., 2019. Natural Heteroplasmy and Mitochondrial Inheritance in Bivalve Molluscs. Integrative and Comparative Biology 59, 1016–1032. https://doi.org/10.1093/icb/icz061

Gillespie, G., 2007. Distribution of non-indigenous intertidal species on the Pacific Coast of Canada. NIPPON SUISAN GAKKAISHI 73, 1133–1137. https://doi.org/10.2331/suisan.73.1133

Hawkins, S.J., Corte-Real, H.B.S.M., Pannacciulli, F.G., Weber, L.C., Bishop, J.D.D., 2000. Thoughts on the ecology and evolution of the intertidal biota of the Azores and other Atlantic islands. Hydrobiologia 440, 3–17. https://doi.org/10.1023/A:1004118220083

Hedrick, P.W., 2007. SEX: DIFFERENCES IN MUTATION, RECOMBINATION, SELECTION, GENE FLOW, AND GENETIC DRIFT. Evolution 61, 2750–2771. https://doi.org/10.1111/j.1558-5646.2007.00250.x

Heller, J., 2015. Marine Ancestors of most Land Snails: Pulmonates, in: Heller, J. (Ed.), Sea Snails: A Natural History. Springer International Publishing, Cham, pp. 257–264. https://doi.org/10.1007/978-3-319-15452-7_10

Herbert, D., 2012. Myosotella myosotis (Mollusca: Ellobiidae) — an overlooked, but well-established introduced species in South Africa. African Journal of Marine Science 34, 459–464. https://doi.org/10.2989/1814232X.2012.716374

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755. https://doi.org/10.1093/bioinformatics/17.8.754

Kaňuch, P., Berggren, Å., Cassel-Lundhagen, A., 2014. Genetic diversity of a successful colonizer: isolated populations of Metrioptera roeselii regain variation at an unusually rapid rate. Ecology and Evolution 4, 1117–1126. https://doi.org/10.1002/ece3.1005

Kawecki, T.J., Ebert, D., 2004. Conceptual issues in local adaptation. Ecology Letters 7, 1225–1241. https://doi.org/10.1111/j.1461-0248.2004.00684.x

Kh Al-Khafaji *et al.*, K., 2021. First record of the invasive snail Myosotella myosotis (Draparnaud, 1801) in coast Shatt al-Arab estuary/ Al-Faw District, Basrah-Iraq. Egyptian Journal of Aquatic Biology and Fisheries 25, 321–328. https://doi.org/10.21608/ejabf.2021.175535

Le Roux, J., Wieczorek, A. m., 2009. Molecular systematics and population genetics of biological invasions: towards a better understanding of invasive species management. Annals of Applied Biology 154, 1–17. https://doi.org/10.1111/j.1744-7348.2008.00280.x

Linetskii, B., Son, M.O., Koshelev, A.V., 2020. Contribution to the knowledge on supralittoral macroinvertebrates of the northwestern Black Sea. Ecologica Montenegrina 34, 8–19. https://doi.org/10.37828/em.2020.34.2

López-Goldar, X., Agrawal, A.A., 2021. Ecological Interactions, Environmental Gradients, and Gene Flow in Local Adaptation. Trends in Plant Science 26, 796–809.

https://doi.org/10.1016/j.tplants.2021.03.006
Marín, C., 2021. Paleobiología del muelle de la Linterna (Portus, Roma). Paleobiología del muelle de la Linterna (Portus, Roma) 635–653.
Mitov, P., 2016. New data on Myosotella myosotis (Draparnaud, 1801) (Pulmonata: Basommatophora: Ellobiidae) from Bulgaria. Acta Zoologica Bulgarica 68, 321–330.
Morton, J.E., Yonge, M., 1997. The functional morphology of the British Ellobiidae (Gastropoda, Pulmonata) with special reference to the digestive and reproductive systems. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 239, 89–160. https://doi.org/10.1098/rstb.1955.0007
Paradis, E., Jombart, T., Kamvar, Z.N., Knaus [aut, B., cph, Schliep, K., Potts, A., Winter, D., 2023. pegas: Population and Evolutionary Genetics Analysis System.
Rijal, D.P., Falahati-Anbaran, M., Alm, T., Alsos, I.G., 2015. Microsatellite Markers for Heracleum persicum (Apiaceae) and Allied Taxa: Application of Next-Generation Sequencing to Develop Genetic Resources for Invasive Species Management. Plant Mol Biol Rep 33, 1381–1390. https://doi.org/10.1007/s11105-014-0841-y
Robbins, A., Griffiths, C.L., 2023. Unusual composition and vertical zonation patterns amongst the biota of pebble and cobble shores. Estuarine, Coastal and Shelf Science 282, 108255. https://doi.org/10.1016/j.ecss.2023.108255
Robicheau, B.M., Powell, A.E., Del Bel, L., Breton, S., Stewart, D.T., 2017. Evidence for extreme sequence divergence between the male- and female-transmitted mitochondrial genomes in the bivalve mollusc, Modiolus modiolus (Mytilidae). Journal of Zoological Systematics and Evolutionary Research 55, 89–97. https://doi.org/10.1111/jzs.12160
Rodrigues, P., Lopes, R.J., Micael, J., Resendes, R., Ramos, J.A., Tristão da Cunha, R., 2014. Genetic and morphometric diversity of the goldcrest (Regulus regulus) populations in the Azores. Zoology 117, 383–391. https://doi.org/10.1016/j.zool.2014.07.001
Romero, P.E., Pfenninger, M., Kano, Y., Klussmann-Kolb, A., 2016. Molecular phylogeny of the Ellobiidae (Gastropoda: Panpulmonata) supports independent terrestrial invasions. Molecular Phylogenetics and Evolution 97, 43–54. https://doi.org/10.1016/j.ympev.2015.12.014
Rumeu, B., Caujapé-Castells, J., Blanco-Pastor, J.L., Jaén-Molina, R., Nogales, M., Elias, R.B., Vargas, P., 2011. The Colonization History of Juniperus brevifolia (Cupressaceae) in the Azores Islands. PLOS ONE 6, e27697. https://doi.org/10.1371/journal.pone.0027697
Sander, K., 1967. Die Gelege von zwei indischen Prosobranchiern der Gattung Assiminea (Leach) Fleming 1828. Z. Morph. u. Okol. Tiere 60, 135–140. https://doi.org/10.1007/BF00403490
Sanford, E., Kelly, M.W., 2011. Local Adaptation in Marine Invertebrates. Annual Review of Marine Science 3, 509–535. https://doi.org/10.1146/annurev-marine-120709-142756
Schaefer, H., Moura, M., Maciel, M., Silva, L., Rumsey, F., Carine, M., 2011. The Linnean shortfall in oceanic island biogeography: A case study in the Azores. Journal of Biogeography 38, 1345–1355. https://doi.org/10.1111/j.1365-2699.2011.02494.x
Schultz, J.A., Hebert, P.D.N., 2022. Do pseudogenes pose a problem for metabarcoding marine animal communities? Mol Ecol Resour 22, 2897–2914. https://doi.org/10.1111/1755-0998.13667
Seelemann, U., 1968. Zur überwindung der biologischen Grenze Meer—Land durch Mollusken. Oecologia 1, 356–368. https://doi.org/10.1007/BF00386689
Sheidai, M., Ziaee, S., Farahani, F., Talebi, S.-M., Noormohammadi, Z., Farahani, Y., 2014. Infra-specific genetic and morphological diversity in Linum album (Linaceae). Biologia 69, 32–39.

https://doi.org/10.2478/s11756-013-0281-4
Śmietanka, B., Zbawicka, M., Sańko, T., Wenne, R., Burzyński, A., 2013. Molecular population genetics of male and female mitochondrial genomes in subarctic Mytilus trossulus. Mar Biol 160, 1709–1721. https://doi.org/10.1007/s00227-013-2223-7
Tigano, A., Friesen, V.L., 2016. Genomics of local adaptation with gene flow. Molecular Ecology 25, 2144–2164. https://doi.org/10.1111/mec.13606
Wickham, H., 2023. plyr: Tools for Splitting, Applying and Combining Data.
Wickham, H., 2022. reshape: Flexibly Reshape Data.