



**Ana Rita Andril
Monteiro**

**Estrutura genética de populações de mexilhão no
NE Atlântico e Mediterrâneo: conectividade entre
habitats do mar profundo**

**Genetic structure of mussel populations in NE
Atlantic and Mediterranean: connectivity between
deep-sea habitats**

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Estrutura genética de populações de mexilhão no NE Atlântico e Mediterrâneo: conectividade entre habitats do mar profundo

Genetic structure of mussel population in NE Atlantic and Mediterranean: connectivity between deep-sea habitats

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica da Doutora Luciana de Melo Santos Génio, investigadora em pós-doutoramento do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro, e coorientação da Doutora Clara Lúcia Ferreira Rodrigues, investigadora em pós-doutoramento do Departamento de Biologia e do CESAM da Universidade de Aveiro, e da Professora Doutora Maria Marina Pais Ribeiro da Cunha, professora auxiliar do Departamento de Biologia da Universidade de Aveiro.



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palavras-chave

Bathymodiolinae, depósitos orgânicos, dispersão larvar, mtCOI, rede de haplótipos

resumo

As espécies persistem ao longo do tempo devido à troca de indivíduos entre subpopulações. No ambiente marinho, a maioria dos organismos bentônicos têm ciclos de vida complexos, envolvendo larvas pelágicas que são transportadas por correntes oceânicas contribuindo para dispersão das espécies. A dispersão larvar estabelece conectividade entre populações geograficamente separadas e afeta a estrutura da população. O conhecimento deste processo biológico promove informações importantes para a conservação de populações marinhas. Este estudo investiga a estrutura genética e conectividade de populações de mexilhão de profundidade entre habitats fragmentados no NE Atlântico e Mediterrâneo. O gene mitocondrial, Citocromo Oxidase I (mtCOI), foi utilizado para analisar diversidade genética por local e a estrutura populacional de duas espécies de mexilhão, *Idas modiolaeformis* e "*Idas*" *simpsoni*. As populações de cada uma das espécies não se encontram geograficamente isoladas. A presença de um haplótipo dominante para cada espécie sugere a partilha de polimorfismos ancestrais entre populações do Mediterrâneo e do NE Atlântico. As populações de *I. modiolaeformis* demonstraram uma elevada diferenciação genética, indicando estruturação da metapopulação. Populações distantes umas das outras, localizadas no Atlântico e E Mediterrâneo, revelaram baixas distâncias genéticas, sugerindo fluxo genético entre as duas regiões. Distâncias genéticas e geográficas suportam o modelo de ilha como o modelo para a estrutura populacional de *I. modiolaeformis*. Uma grande desvantagem deste estudo está relacionada com o número discrepante de indivíduos entre populações. Para investigar os padrões de conectividade em diferentes escalas espaciais serão necessários mais estudos, utilizando mais espécimes e outros marcadores genéticos.

keywords

Bathymodiolinae, organic falls, larval dispersal, mtCOI, haplotype network

abstract

Species persist over time, due to exchange of individuals between subpopulations. In the marine environment, most benthic organisms have complex life cycles including pelagic larvae that are transported by ocean currents promoting species dispersal. Larval dispersal connects geographically distant populations and determines population structure. The knowledge about this biologic process provides relevant information for conservation of marine populations. This study investigates the genetic structure and connectivity of deep-sea mussel populations between fragmented habitats in the NE Atlantic and Mediterranean. The mitochondrial Cytochrome Oxidase I (mtCOI) gene was used to analyze site-specific genetic diversity and the population structure of two mussel species, *Idas modiolaeformis* and "*Idas*" *simpsoni*. Populations of each species are not geographically isolated. The presence of one dominant haplotype for each species suggests shared ancestral polymorphisms between Mediterranean and NE Atlantic populations. The overall high genetic differentiation observed in *I. modiolaeformis* indicates that the metapopulation is structured. Distant populations, located in Atlantic and E Mediterranean, revealed low genetic distances, suggesting gene flow between the two regions. Genetic and geographical distances support an *island* model of *I. modiolaeformis* population structure. A major drawback of this study is concerned with the discrepant number of individuals among populations. Further research will be needed, using more specimens and other gene markers, to investigate connectivity patterns at different spatial scales.

“Os mexilhões *xom xom xom xom xom xom xom* os mesmos”
Autor desconhecido

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INTRODUCTION

The term biological conservation emerged in the 80s, responding to threats and losses of biodiversity. The goal of this discipline is to understand, protect and perpetuate biological diversity among all scales and levels of biological organization, so present and future generations can sustainably explore and use them. Nowadays, conservation brings together ecological, environmental, social, economic and political fields, among other disciplines (Meine, 2010). Prevention measures require the study of species distribution, abundance and ecological functions, and evaluation of risks and threats for species conservation (reviewed in Goodsell and Underwood, 2009).

Regarding the marine environment, conservation of species or habitats is achieved through establishment of marine protected areas (MPAs) and legislation (restriction, regulation or prohibition of habitats destruction or modification). MPAs range from totally closed to any anthropogenic activity, to limited catches or temporary closures to human actions. The implementation of marine reserves restores biodiversity of endangered species or important habitats and increases fisheries, creating long-term economic benefits. However, in a short term, measures of conservation generate conflict with economic interests. Thus, design and management of MPAs should integrate not only ecological concerns, but also economic, political and social issues (reviewed in Goodsell and Underwood, 2009).

Corridors between reserves were proved to be extremely important, once they allow exchange of individuals/genes preventing extinction of populations due to inbreeding depression. For many marine populations, ocean currents serve as corridors for organisms dispersal (reviewed in Goodsell and Underwood, 2009). Genetic connectivity between MPAs is an important factor to the maintenance of MPA networks (Bell, 2008). For this reason, knowledge about hydrodynamics, dispersal and connectivity between populations is important to the management of MPAs.

Dispersal, settlement and recruitment are biological processes extremely important to the ecology (geographical distribution, abundance) and evolution of marine populations (reviewed in Jenkins et al., 2009; Marshall et al., 2009). Larval dispersal is inferred from gene flow between populations and population structure. Recently, molecular genetic markers (indirect approaches) have been used to estimate gene/larval exchange between populations (Marshall et al., 2009).

Population structure and connectivity research provides important data, for example inferences about source-sink dynamics, habitat's ability for recolonization, among other

relevant information (Boschen et al., 2015; Marshall et al., 2009), which is crucial to conservation of marine populations (Thaler et al., 2014; Marshall et al., 2009).

This dissertation intends to increase the knowledge about population structure and connectivity that will be useful to advice on legislation and management strategies for marine habitats. In this study, I used a genetic approach to investigate the population structure of two deep-sea chemosymbiotic mussels (*Idas* spp.), providing new insights into population connectivity among fragmented habitats in the NE Atlantic and Mediterranean area. Following is a description of the chapters' content:

Chapter 1 reviews concepts of population structure and connectivity, which in organisms with a larval stage is maintained by larval supply into the environment, larval dispersal to other populations, and also settlement and recruitment in new habitats. This study addresses deep-sea populations, thus deep-water ecosystems were briefly characterized, with a particular focus on reduced habitats, inhabited by chemosymbiotic mussels, followed by an overview of the main threats to these habitats and examples of deep-ocean MPAs. Finally, knowledge about deep-sea mussels was reviewed, specifically the Bathymodiolinae subfamily, indicating reproductive adaptations to reduced habitats.

Chapter 2 presents the framework of this project, including recent studies on population genetic structure and connectivity, and a brief mention to the CHEMECO project that provided samples for this study. The objectives of this work are defined.

Chapter 3 describes the geology and hydrodynamics of the study region and characterizes the habitats where species were found. Methods used in sampling collection, DNA sequencing and data analyses are described. The dataset (including available GenBank sequences) used in this investigation is provided.

Chapter 4 presents the results, namely the success rate of DNA extractions, amplifications, and sequencing, followed by the phylogenetic reconstruction and population structure analyses, including haplotype network, AMOVA, SAMOVA, pairwise genetic distances, and lastly, correlation analysis between geographic and genetic distances.

Chapter 5 discusses the data obtained in this study in comparison with other studies, and its interpretation.

Chapter 6 refers the main conclusions and limitations of this study, and some improvements are suggested for future research, as well as further methods for investigating population structure and connectivity in the deep sea.

Chapter 1. LITERATURE REVIEW

1.1. Population structure and connectivity

The exchange of individuals between populations is crucial for the persistence of populations. Over time, a population prevails when the number of births and immigration are superior or equal to the number of deaths and emigration (Cowen and Sponaugle, 2009; Lowe and Allendorf, 2010). If the population does not have migrants neither entering nor leaving the population, yet births are superior to deaths, this population is considered as *closed*. In this case, problems deriving from inbreeding may occur, such as infertility or loss of viability, due to matting between genetically closely-related individuals (Allendorf and Luikart, 2007; Cowen and Sponaugle, 2009; Gillespie, 1998). If the population exchanges (receive and export) individuals with other populations, it is considered as an *open* population. The random exchange between open populations is known as panmixia (Cowen and Sponaugle, 2009; Hellberg et al., 2002).

Most natural populations cannot behave as a single randomly mating population, because species can occupy vast geographical areas or have barriers to migration (Gillespie, 1998). Natural populations are generally subdivided or structured in smaller units, where local random matting may occur (Allendorf and Luikart, 2007). The largest population can be referred as metapopulation and the units can be named populations or subpopulations. Hence, metapopulation is defined as an “assemblage of discrete local populations with some measure of shared migration among them” (Cowen and Sponaugle, 2009; pg 444).

The metapopulation structure is expected to reflect genetic variation within and between local populations (Allendorf and Luikart, 2007). The study of population genetic structure investigates which evolutionary forces (migration, genetic drift or natural selection) are responsible for the genetic variation across a geographic area (Allendorf and Luikart, 2007; Gillespie, 1998). According to the Hardy-Weinberg principle, the transmission of genetic information over generations occurs without mutation (locus equilibrium) in a randomly mating population without evolutionary forcing, such as genetic drift, natural selection and migration. Thus, genetic differentiation between populations indicates deviations to the Hardy-Weinberg equilibrium (Gillespie, 1998).

Empirical models of metapopulation structure vary according to population size and isolation (Aycrigg and Garton, 2014). Considering the complete isolation of the populations, one can make two assumptions: random mating and genetic drift in each isolated

subpopulation. Over time, each subpopulation will fix one or more alleles. However, migration usually occurs which implies gene flow among populations. The most simplified case is to consider two equal sized populations with bidirectional exchange of individuals. We can consider gene flow with or without genetic drift. When isolated populations are subject to gene flow and genetic drift, populations will be genetically distinct (Allendorf and Luikart, 2007).

Gene flow tends to genetically homogenize the individuals of geographically separated populations but, on the other hand, genetic drift and natural selection cause divergence within a metapopulation. Neutral evolution attributes the genetic variation within a population to migration and genetic drift, excluding natural selection. Therefore, neutral gene divergence between populations will be a balance of gene flow (due to dispersal) and genetic drift (Allendorf and Luikart, 2007; Hartl and Clark, 1997).

Based on different patterns of gene flow, several models are used to describe the structuring of populations (Figure 1).

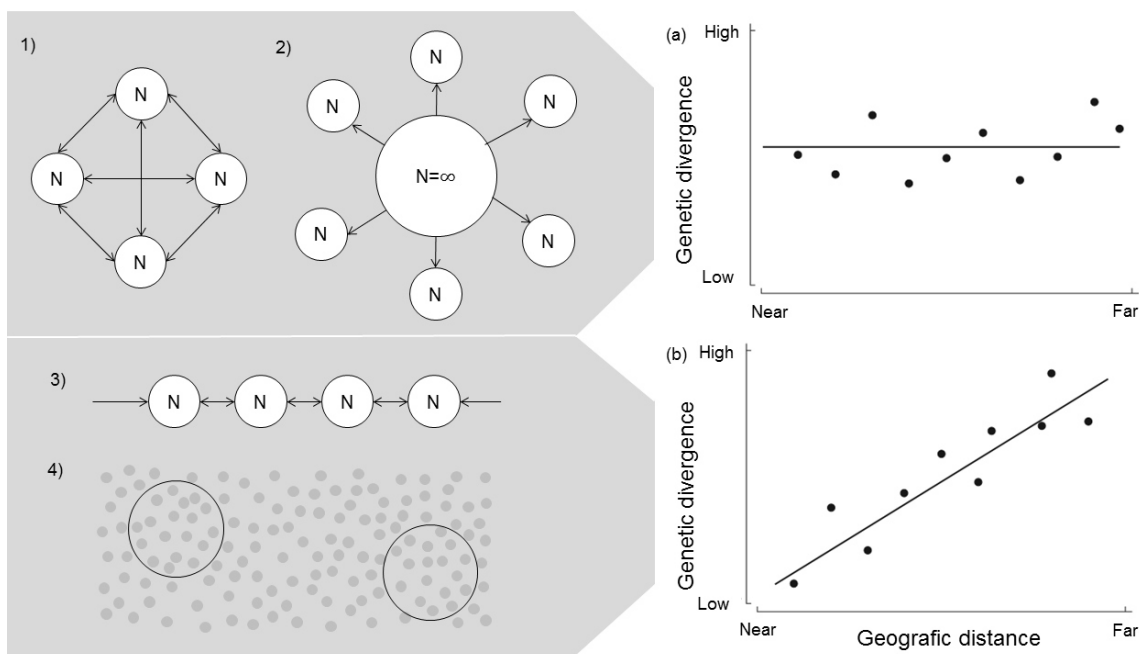


Figure 1 – Models of population structure (1-4) and expected correlations between genetic and geographic distances (a, b). *Island* model with two versions 1) the classical and 2) the *mainland-island*; 3) *stepping-stone* model and 4) *isolation-by-distance* model. Circles represent populations and arrows represent gene flow. Expected correlations between genetic divergence and geographic distances are exhibited for: (a) *island* model and (b) *stepping-stone* and *isolation-by-distance* models. Images adapted from Allendorf and Luikart (2007) and Altukhov (2006).

The *island* model has two versions. In the first version, populations have equal size and equal rate of gene exchange (classical). All populations contribute to a common gene pool

where mating occurs randomly. In the second version (mainland-island), one largest panmictic population exists as well as few smaller populations (islands) that receive genes from the mainland. *Isolation-by-distance* model suggests that genetic distances depend on the distance between neighboring populations and on the size of the population (Altukhov, 2006). The *stepping-stone* model represents one intermediate situation between the *island* model and the *isolation-by-distance* model. The *stepping-stone* structure assumes that the exchange of genes only occurs between the neighbor populations, considering equal size of populations and equal migration rates (Altukhov, 2006).

The interchange among geographically distant (sub)populations contributes to metapopulation connectivity (Cowen and Sponaugle, 2009). In this way, studies about population structure allow inferences about connectivity. Population connectivity can be viewed from two perspectives: demographic connectivity and genetic connectivity. Demographic connectivity can be defined as the degree to which population growth and vital rates (birth and survival) are affected by dispersal (immigration or emigration rates) (Lowe and Allendorf, 2010). Genetic connectivity depends on the absolute number of dispersers (emigration) among populations and can be defined as the “degree to which gene flow affects evolutionary processes within subpopulations” (Lowe and Allendorf, 2010). Genetic methods are most useful to infer genetic connectivity, but provide little information on demographic connectivity.

Most marine benthic species have complex life cycles including sessile/sedentary adults and a pelagic larval phase that is the main responsible for species dispersal (Thorson, 1950). Marine population connectivity includes the dispersal phase from reproduction to habitat selection, metamorphosis and complete settlement processes (Figure 2) (reviewed in Cowen and Sponaugle, 2009).

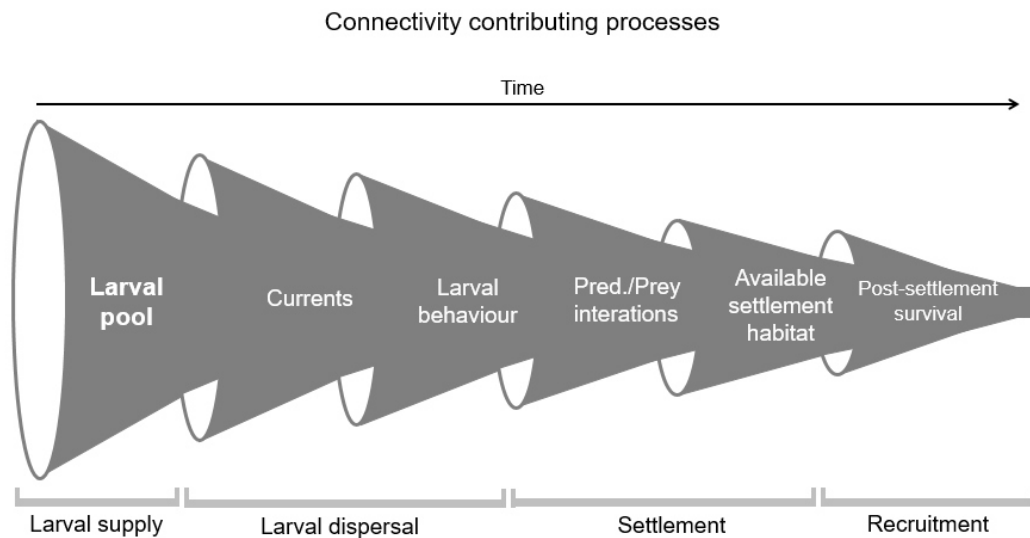


Figure 2 – Connectivity contributing processes: larval supply and dispersal, settlement and recruitment. Dimension of funnel indicates larval abundance that decreases from reproduction to recruitment as a result of physical and biological factors. Adapted from Cowen and Sponaugle (2009) and Pineda (2000).

1.1.1. Larval supply and dispersal

The exchange of individuals, and therefore genes, is related with the rates of dispersal between populations (Allendorf and Luikart, 2007). Supply of larvae is highly variable, influencing strongly both ecology and evolution of marine taxa (reviewed in Marshall et al., 2009). Self-recruitment (reproduction inside a subpopulation) also occurs, mainly in species from patchy habitats, due to the low probability of finding suitable habitats to settle (Cowen and Sponaugle, 2009; Swearer et al., 2002).

The production of larvae depends on fecundity (ability of gametes production) and fertilization success (zygotes production). Fecundity is influenced by intrinsic and extrinsic factors. On one hand, nutritional history, maternal size and age are intrinsic factors that restrict gametes production. On the other hand, environmental stress, intra- and inter-competition can represent some extrinsic factors that regulate fecundity (Ramirez-Llodra, 2002). Variation of fecundity results from egg quantity and quality, for instance, the eggs' dimensions, which affect fertilization success (larger female gametes have better chances of being fertilized) (Gaudron et al., 2012).

In the majority of marine animals fertilization is external, meaning that larval production is not reflected by fecundity (Gaudron et al., 2012). Fertilization success has great fluctuations, rarely reaching 100%. The probability of female and male gamete encounters, gametes quality, local hydrodynamics, density population and intra-specific competition are some factors that affect fertilization success (reviewed in Marshall et al., 2009).

Dispersal processes depend on physical and biological factors. Physical factors consist of currents and turbulence that mix larvae, allowing the expansion or limiting their spatial distribution. Biological factors include larval behaviour (vertical migration, swimming) and predator/prey interactions (Cowen and Sponaugle, 2009; Marshall et al., 2009). Some environmental features may influence larval behaviour (Cowen and Sponaugle, 2009). Sameoto and Metaxas (2008) showed that larval physiological tolerance to temperature and salinity influence vertical distribution, affecting adult population distributions, genetic flow and dispersal movements.

Mortality of dispersing larvae results from physical, environmental and biological factors, for example ocean currents, pollution, salinity changes, availability of suitable settlement habitat, predation and post-settlement survivorship. The mortality during the planktonic period affects the dispersal distance and the quantity of recruited larvae into a population (Cowen and Sponaugle, 2009; Marshall et al., 2009).

Pelagic Larval Duration (PLD) is the time that larvae spend dispersing in water column (Shanks et al., 2003). Species phylogeny and environmental features determine PLD. Thus, PLD can be season-, species- and location-specific (Cowen and Sponaugle, 2009; Hilário et al., 2015). Hilário and other authors (2015) reviewed the knowledge about PLD, concluding that PLD vary with bathymetric range of benthic organisms. Shallow organisms (depth<200 m) have a shorter and less variable PLD (25-30.35 days), whereas deep-sea organisms (depth>200 m) have a larger and more variable PLD (73.40-96.63 days). This measure may be used to estimate dispersal distances, when it is coupled with advection velocity. Dispersal distances estimated for several taxa range from a few meters to 1000 kilometres (Cowen and Sponaugle, 2009). However, this approach is limited, since dispersal processes relate not only with the larvae pelagic time, but also involve larval behaviour and hydrodynamic processes. Therefore, the use of biophysical models, incorporating physical features (temperature, salinity, velocity of oceanic currents, etc) with biological features (larva's feeding regime, larval behaviour, PLD, etc), are crucial to estimate dispersal distance (reviewed in Hilário et al., 2015).

1.1.2. Settlement and recruitment

The free-swimming larval stage (dispersal) culminates on the settlement of competent individuals (reviewed in Cowen and Sponaugle, 2009). Settlement processes involve the contact of larvae with substrate, the exploration and inspection, and also the acceptance or rejection of the site. Thus, settlement usually represents the end of the pelagic period and

the initiation of the sedentary life. Larval behaviour, regulated by physical and biological cues, allows larvae to choose between accepting and rejecting the settlement site. When larvae reject the settlement site, they are released from the substrate returning to the water column. When larvae accept the site, settlers attach to the substrate and then metamorphosis occurs. To ensure that settlement occurs in an appropriate environment (that allow larval survival, growth and reproduction) larvae respond to a set of cues that stimulates settlement behaviour. Some examples of biological cues are food supply, microbial film and presence of conspecifics (potential mates), competitors and predators. Physical cues include availability of space, rock type and mineral composition, micro-topography, temperature, salinity, hydrostatic flow and pressure, among others. Settlement processes lead to a high mortality rate, since the larvae suffer considerable morphological changes at the metamorphosis (reviewed in Jenkins et al., 2009).

The term recruitment lies ill-defined, however it is considered a combination of settlement and post-settlement period, when growth and reproduction occur. Recruitment pattern can be influenced by density-dependent interactions or by the nutritional requirements at the benthic stage. Recruitment shows spatial and temporal variability due to environmental conditions and intraspecific competition (for food and space) (reviewed in Jenkins et al., 2009).

1.2. Deep-sea ecosystems

Oceans cover approximately 70% of the earth's surface and a large portion (~ 50%) is below 3000 m depth (Tyler, 2003). The deep sea is defined from the beginning of the shelf break at approximately 200 m depth and comprises 95% of the volume of the biosphere (Danovaro et al., 2014; Thistle, 2003). Its exploration began in the late 19th century, revealing over the past decades several new geological features (e.g. hydrothermal vents and cold seeps) and highly abundant biological communities (Tyler, 2003).

The vast deep-sea habitat is found in an environment considered relatively stable, characterized by high pressures, low food input and oxygen concentration, and generally low temperatures. The temperature decreases with increasing depth, except at hydrothermal vents, where high temperatures (260-400°C) can be reached (Fisher et al., 2007; Thistle, 2003). On the abyssal plain the temperature reaches -2°C to 2°C (Thistle, 2003). Erosional and depositional processes lead terrigenous, intertidal and subtidal organic material to become coastal particulate organic matter (POM). Small particles (e.g. remains of plankton, fecal pellets, etc), large animal carcasses and plant detritus sink until

reaching the deep-sea floor, turning into POM. Also, dissolved organic matter represents an organic input into the deep sea. Contrasting with usually slow near-bottom water currents, occasional periods of fast flow (e.g. benthic storms and dense shelf water cascades) increase the horizontal food flux and consequently benefit some species (Gage, 2003).

In addition to temperature drop, increasing depth causes an abrupt light intensity decrease. Below ~250 m depth, light penetration can no longer support photosynthesis. An alternative process of primary production is through chemosynthesis. This is one of the particular features that differentiates some deep sea habitats (reviewed in Thistle, 2003).

1.2.1. Reducing habitats

Hydrothermal vents, cold seeps, whale and wood falls are chemosynthesis-based ecosystems with primary production being guaranteed by chemoautotrophic bacteria (Distel et al., 2000; Duperron et al., 2008; Fisher et al., 2007; Smith and Baco, 2003). These chemoautotrophs produce organic compounds using geochemical energy generated from the oxidation of reduced compounds, such as sulphate and nitrate (Duperron et al., 2008; Fisher et al., 2007). The chemosynthetic bacteria support the microbial ecosystem and the animal communities establishing symbiotic associations (Fisher et al., 1994 in Fisher et al., 2007; Duperron, 2010). The term symbiosis used in this context refers to the “close association between a metazoan host and bacteria allowing the host to gain novel metabolic capabilities” (Douglas, 1994 in Duperron, 2010; pg 138).

In hydrothermal vents that are usually located on active tectonic plate boundaries (Figure 3), the water is heated and enriched in reduced compounds such as sulphide and metals. The interactions between the geo-thermally heated fluids and cold seawater precipitate the dissolved minerals and metals forming chimneys (Duperron, 2010). Hydrothermal vent communities include a great diversity of invertebrate phyla, such as Annelida, Mollusca, Crustacea, among others. Octopuses and fishes also occur near this habitat (Fisher et al., 2007; Tunnicliffe et al., 1998). In contrast, cold seeps occur mostly along continental margins (Figure 3). In cold seeps, the organic matter is reduced by thermogenesis and biogenesis, producing hydrocarbon and methane-rich fluids that are released to the water column (Duperron, 2010). At these habitats, small bivalves are abundant (particularly families: Mytilidae, Vesicomidae, Thyasiridae), and siboglinid polychaetes are also present (Cunha et al., 2013a; Hilário et al., 2011; Hilário et al., 2010; Olu-Le Roy et al., 2004; Rodrigues et al., 2008).

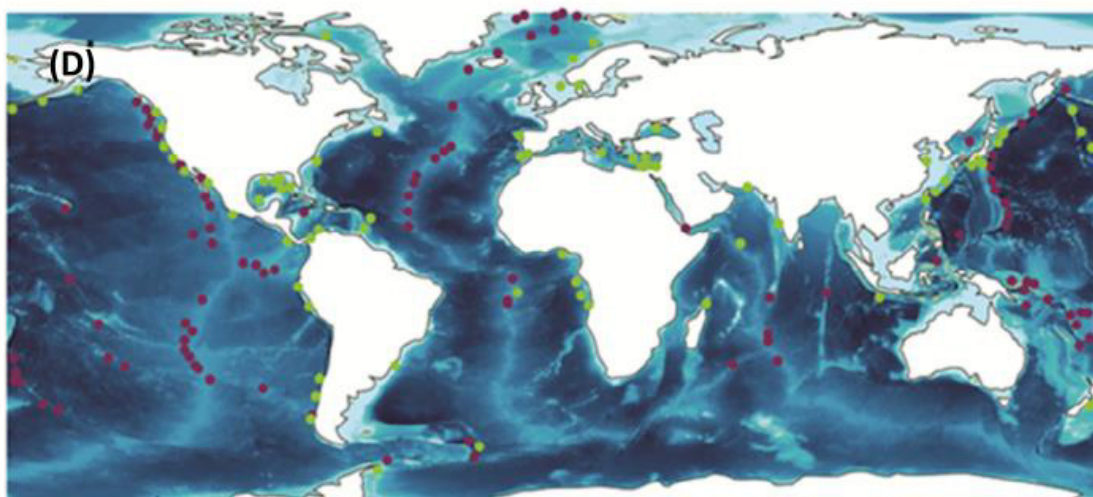


Figure 3 – Distribution of hydrothermal vents (violet) and cold seeps (green) (Danovaro et al., 2014).

In addition to vent and seep habitats, organic falls are also reduced habitats. Submerged mammal skeletons, wood, kelp, seagrass and other organic deposits are ephemeral (short-lived) and fragmented (because organic flux is intermittent in space) habitats (Smith and Baco, 2003; Tunnicliffe et al., 2003). Sunken bones have sulphide-reducing bacteria that reduce lipids into hydrogen sulphide (Smith and Baco, 2003). The product of bacterial decomposition in sunken wood is also sulphide (Distel et al., 2000). The organic decomposition produces reduced compounds (reviewed in Tunnicliffe et al., 2003), enabling chemosymbiotic and other species to settle (Smith and Baco, 2003; Tunnicliffe et al., 1998). Indeed, deep-sea organisms have a rapid and vigorous response to the sunken wood input. For instance, wood is colonised mainly by specialized molluscs, polychaetes and occasionally vestimentiferan tube worms (Turner, 1973; Gage, 2003; Tunnicliffe et al., 2003).

The availability of habitat for settlement, such as organic falls, can reduce dispersal distances of organisms. Owing to this fact, organic falls have an important role in deep-sea ecology and in biogeographic distribution of chemosymbiotic species (Génio et al., 2014). Interestingly, some taxa may occur in vents, seeps, whales and wood falls (Tunnicliffe et al., 1998). The taxonomic overlap between these habitats suggested that organic falls can serve as “dispersal stepping stones” for seep and vent habitats (Smith and Baco, 2003; Tunnicliffe et al., 1998).

1.2.2. Threats to deep-sea habitats

Deep-sea habitats as well as other marine ecosystems are threatened. Climate change, pollutant contamination (Figure 4A and AB), debris, over-harvesting and habitat loss are some examples of such threats. In some cases, the impact on the habitats are more intensive, in others are more extensive, leading to different consequences for biota (reviewed in Goodsell and Underwood, 2009).

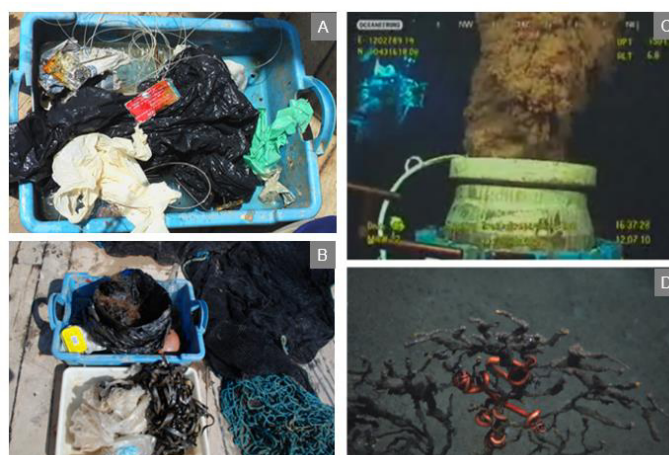


Figure 4 – Example of anthropogenic impacts in deep sea: litter collected from Mediterranean at 1200 m (A) and at 3000 m in Pacific Ocean (B); oil discharge in water column in Gulf of Mexico (C) and deep-sea coral covered with oil in Gulf de Mexico (D). Images from Ramirez-Llodra et al. (2011).

Metals and minerals are widely extracted from land and onshore reserves. However, as these resources are being depleted, exploration is moving to deeper, offshore areas. Manganese nodules, cobalt crusts and massive sulphides are important deposits of metals in the deep-sea (depths > 4000 m) and have great economic relevance. Nowadays, sea-floor mining is still very expensive, but underwater mining equipment is being developed, and environment impact of this activity is still uncertain (Lange et al., 2014). Some ecological studies have been conducted in order to minimize the exploration impacts (Vanreusel et al., 2016).

Similar to mineral exploration, the great oil and gas consumption lead to depletion of shallow-water reserves, driving the exploration of these resources at deeper depths (Lange et al., 2014). The exploration of oil and gas has enormous consequences to the marine environment (Figure 4C and 4D), resulting from routine activity or accidental contamination from extraction stations. Some of the routine activity impacts include: excess of sedimentation, chemical contamination of the water, damage of hard substratum (such as coral reefs), as well as light and sound pollution. Accidental impacts resulting from oil and

gas release are: contamination of water, fauna mortality and disturbance of marine community (reviewed in Cordes et al., 2016).

Marine protected areas (MPAs) have been implemented to preserve biodiversity and habitats (reviewed in Goodsell and Underwood, 2009). In the last decades, concerns about deep-sea habitats and biodiversity are increasing, and a few MPA have already been established in deep water. The first deep-sea habitats protected were hydrothermal vents, the Lucky Stricke and Menez Gwen vent fields (Azores) in 2002 and Endeavour Segment (Canada) in 2003 (Leary, 2006; Vrijenhoek, 2010).

Aiming to the management of the manganese nodules mining at Clarion-Clipperton Zone (Pacific Ocean), the International Seabed Authority (ISA) assigned in 2012 areas of particular environmental interest (APEI's), prohibiting mineral extraction in a total area of 160000 km² (Lange et al., 2014).

Recently, July of 2015, the Portuguese government declared the seamounts of the Gorringe Bank as marine protected area, with depths ranging from 25 to 5000 m. The area proposed to conservation has 2 288 782.11 hectares - *Resolução do Conselho de Ministros* n.º 59/2015 (Diário da República, 2015).

1.3. Deep-sea mussels – close look to Bathymodiolins (Bivalvia: Mytilidae)

Over the years, several species of mussels have been reported worldwide from 200 to 3600 m depth. About 37 species have been found associated to reduced environments including vents, seeps and organic falls (e.g. whale carcasses or sunken wood) (Baco and Smith, 2003; Distel et al., 2000; Duperron, 2010; Smith and Baco, 2003). However, more hidden diversity have recently been found based on molecular research (Faure et al., 2015; Génio et al., 2014; Lorion et al., 2009).

The remarkable difference between deep-sea mussels and their coastal relatives is the occurrence of chemosynthetic bacterial endosymbionts (Duperron et al., 2008; Duperron, 2010). Regarding the Mytilidae family, all subfamilies are assymbiotic except for the Bathymodiolinae subfamily, containing chemosymbiotic species (Taylor and Glover, 2010). Moreover, mytilids have an important role in the deep-sea ecosystem, providing hard substratum (ecosystem engineers), available food sources such as fecal pellets (for detritus feeders) and dead remains (for scavengers) (Turner, 1973).

The origin of the Bathymodiolinae subfamily was registered between the Cenozoic and the late Mesozoic. Bathymodiolins have a pattern of evolution from shallow (<1000 m) to deep habitats, with multiple re-radiations in deeper waters (Jones et al., 2006; Lorion et al.,

2013). These authors suggested that shallow-water cold seeps, hydrothermal seamounts and hydrocarbon deposits may have served as a refuge during extinction events (anoxic/dysoxic events) in deep-sea environments. Hence, these refuges provided opportunities to new evolutionary radiation into deep waters. Furthermore, it was proposed that seep and vent mussels derived from ancestors associated with wood and other organic-falls habitats, proving that deep-sea mussels took “wooden steps to deep-sea vents” (Distel et al., 2000; Lorion et al., 2013). This supports the hypothesis of multiple events of vent and seep colonization across geological time (Distel et al., 2000; Duperron, 2010; Lorion et al., 2013).

Over time, this evolution from organic-enriched habitats to vent/seep habitats was unidirectional, indicating an irreversible specialization mainly due to anatomical and physiological adaptations (Lorion et al., 2013). It is possible to distinguish two main groups within bathymodiolins: mussels with larger shells living in hydrothermal vents and cold seeps (genus *Bathymodiolus*, *Gigantidas* and *Vulcanidas*) and smaller mussels that exist predominantly in sunken organic substrates (genus *Idas*, *Adipicola* and *Benthomodiolus*) (Lorion et al., 2013; Smith et al., 1998; Smith and Baco, 2003; Taylor and Glover, 2010; Turner, 1973).

The main adaptations of mussels to chemosynthetic habitats are related with feeding and reproductive strategies (Le Pennec and Beninger, 2000). Most bathymodiolins harbor sulphur-oxidizing and/or methane-oxidizing symbionts in their gill epithelial cells (Duperron et al., 2008; Duperron, 2010; Fisher et al., 1987; Laming et al., 2015a). This relationship between bacteria and mussels are probably the key for the success of mussels in chemosynthetic environments (Duperron, 2010). Sulphur-oxidizing symbionts occur in various species, opposed to methane-oxidizing symbionts that are extremely rare among bivalves (Duperron, 2010; Taylor and Glover, 2010). The host-symbiont relationship is not only characterized by the symbiont type (sulphur or methane-oxidizing), but also by the symbiont abundance and diversity (Duperron et al., 2008; Laming et al., 2015a; Laming et al., 2015b; Rodrigues et al., 2012). All these features strongly influence the species ecological strategies. For instance, high symbiont flexibility (ability of harbor high symbiont diversity) indicates the use of diverse energetic sources (Gaudron et al., 2012). Chemosymbiotic mussels did not lose the capability of filter feeding, however they need reduced compounds to survive, so they have a mixotrophic feeding strategy (Duperron, 2010).

Furthermore, reproductive strategies including reproductive cyclicity, larval stage, trophic strategy and rate of larval development, growth rate, maturation period, among other

features, also contribute to the ecological success of this subfamily in deep-sea chemosynthetic habitats (Le Pennec and Beninger, 2000; Turner, 1973; Tyler et al., 2007; Tyler et al., 2009).

1.3.1. Reproductive adaptations

The majority of bathymodiolins are dioecious or successive hermaphrodites (Le Pennec and Beninger, 2000; Tyler et al., 2009). For instance, the species *Idas modiolaeformis*, a small bathymodiolin is a protandric hermaphrodite, which means that in the early adult life the individual is male, then becomes hermaphroditic and in late adulthood the individual turns into female (Gaudron et al., 2012; Laming et al., 2014; Tyler et al., 2009).

Reproductive cyclicity appears to depend on the degree of endosymbiosis and presence of environmental cues, such as organic matter flux and temperatures (Le Pennec and Beninger, 2000; Tyler et al., 2007). In habitats with few or without environmental cues species can have a discontinuous reproductive activity (mixotrophic species) or continuous reproductive activity (species with obligate symbionts). In habitats where environmental cues exist, discontinuous reproductive activity is detected (reviewed in Le Pennec and Beninger, 2000). For example, the cold-seep species "*Bathymodiolus*" *childressi* has periodic gametogenic cycles (Arellano and Young, 2009).

The acquisition of larval pelagic stage was another relevant adaptation to colonization of patchy habitats (Tyler et al., 2007). The trophic strategy of larvae and the larval development rate can be inferred from the oocyte size. Deep-sea bivalves with small oocytes are planktotrophic or exotrophic (feeding larvae) and bivalves with larger oocytes are lecithotrophic or endotrophic (Arellano and Young, 2011; Danovaro et al., 2014; Le Pennec and Beninger, 2000). Larval development rate depends on food availability and once again oocyte dimensions. Usually, larger oocytes have faster rates and *vice-versa*. The trophic strategy of larvae influences larval duration and dispersal strategy (reviewed in Le Pennec and Beninger, 2000). Gaudron et al. (2012) suggested a pelagic larvae duration of 4-5 months for the mussel *I. modiolaeformis* and also inferred that the larval development is likely planktotrophic. "*Idas*" *simpsoni* has also planktotrophic larvae (Laming et al., 2015a). "*Bathymodiolus*" *childressi* has a longer PLD compared to smaller bathymodiolins, as they are able to remain more than 13 months in the plankton. The occurrence of a planktotrophic larva was also demonstrated for this genus (Arellano et al., 2014; Arellano and Young, 2009).

Lastly, growth and maturation rates relate with reproductive strategies. The rapid development to achieve reproductive maturity may indicate the species adaptation to patchy and ephemeral habitats (Gaudron et al., 2012). *Bathymodiolus* species have an estimated growth rate of 0.048 mm *per* day (Arellano and Young, 2009). "*Bathymodiolus*" *childressi* is considered mature with shell sizes ranging between 50-120 mm (Arellano and Young, 2009), corresponding to a maturation age of about 3 years (shell length: 50 mm) (Rhoads et al., 1981). *Idas* growth rate ranges between 0.017 and 0.029 mm *per* day, with the first maturation being reached at 2-4 months of age (shell length: 2-2.35 mm) (Génio et al., 2014; Laming et al., 2015a; Laming et al., 2015b).

Chapter 2. FRAMEWORK AND OBJECTIVES

Population genetic structure is based on the study of DNA variation in natural populations. Several methods are used to detect variation in DNA, from mitochondrial DNA to nuclear DNA, including single base polymorphisms (SNPs), microsatellites, and others.

In the current study, mitochondrial DNA was chosen due to a number of characteristics that allow assessing patterns of genetic variation: 1) it is haploid; 2) in most species, it is maternally inherited; 3) it does not undergo recombination and 4) for most metazoan taxa it evolves rapidly, showing recent divergence events (Allendorf and Luikart, 2007; Cowen and Sponaugle, 2009; Hartl and Clark, 1997). For deep-sea mytilids, the mitochondrial DNA Cytochrome Oxidase subunit I (mtCOI) gene is assumed to diverge at a rate of $\approx 1\text{-}2\%$ *per* Myr (Lorion et al., 2012; Won et al., 2003).

Several studies to assess deep-sea population connectivity and population structure have been conducted recently using the mtCOI gene. For instance, population structure and connectivity of the shrimp (*Chorocaris* sp. 2) and the squat lobster (*Munidopsis lauensis*) (Thaler et al., 2014); interpopulational relationships of *Bathymodiolus septemdierum* complex in Indo-Pacific deep-sea vents (Breusing et al., 2015); distribution and population connectivity of several *Bathymodiolus* species occurring in the Gulf of Mexico cold seeps (Faure et al., 2015). Few genetic connectivity studies have integrated both Atlantic and Mediterranean areas (Affinito et al., 2015; Andreakis et al., 2009; Correia et al., 2012; Griffiths et al., 2011). Most studies were focused on either the Atlantic (Bradbury et al., 2014; Cuveliers et al., 2012; Pita et al., 2016; Ribeiro et al., 2010) or the Mediterranean basins (Calò et al., 2016; Casado-Amezúa et al., 2012; Félix-Hackradt et al., 2013; Garofalo et al., 2009; Maggi and González-Wangüemert, 2015; Pilczynska et al., 2016; Schiavina et al., 2014). In general, the most commonly studied organisms are fishes, whereas genetic connectivity of deep-sea chemosynthesis-based populations has not been investigated in this region.

The deep-sea mussel species found in the Gulf of Cadiz hold a great research potential for speciation and divergence over the Atlantic and connectivity in the Eastern Mediterranean seeps (Rodrigues et al., 2012). In this work, two deep-sea mussel species were used as study organisms: *Idas modiolaeformis* (Sturany, 1896) and “*Idas*” *simpsoni* (Marshall, 1900) (Figure 5). *Idas modiolaeformis* (also named *Idas* sp. *Med*) has only been recorded in cold seep habitats in the Eastern Mediterranean (Duperron et al., 2008), western Mediterranean and Atlantic Ocean (Laming, et al., 2015b; Rodrigues et al., 2012). Unlike the usual dense mussel aggregation, *Idas modiolaeformis* occurs in low densities of

individuals and rarely conspecifics have direct contact (Gaudron et al., 2012; Laming et al., 2015a). “*Idas*” *simpsoni* occurs on carbonates nearby seeps and bone-fall substrata, forming several small clumps (Génio et al., 2014; Laming et al., 2014). The taxonomy of this last species is under discussion, Thubaut and other authors (2013) proposed that “*Idas*” *simpsoni* could be included in the genus *Nypamodiolus*. Because this name has not been formally accepted, I continue to use the generic name *Idas* but with quotation marks.

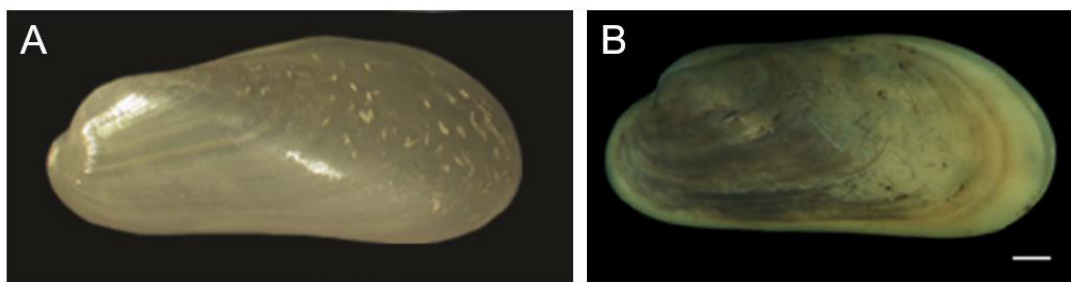


Figure 5 – *Idas modiolaeformis* (A), 11 mm (Génio, 2009) and “*Idas*” *simpsoni* (B), scale bar: 1 mm (Génio et al., 2014).

Samples of *I. modiolaeformis* were obtained within CHEMECO project. This project investigated the process of colonization in reduced deep-sea habitats along the NE Atlantic and Mediterranean. The sampling design consisted on deployment and later recovery of colonization devices with different types of substrates (wood, alfalfa grass and carbonates) named CHEMECOLI - CHEMosynthetic Ecosystem COLonization by Larval Invertebrates (Cunha et al., 2013b; Gaudron et al., 2010). *Idas modiolaeformis* mussels were found in abundance in the wood and alfalfa substrates deployed in Meknès and Darwin mud volcanoes in the Gulf of Cadiz. Further specimens of this species were later collected during a research cruise in Gorringe Bank on a wood log (Rodrigues et al., 2012). MtCOI sequences of *Idas modiolaeformis* from Mediterranean sites are available in GenBank, allowing to expand my NE Atlantic dataset and investigate population structure in a larger geographic area.

After molecular analyses I found “*Idas*” *simpsoni* specimens that have been mistakenly identified as *I. modiolaeformis* based on morphological traits. The two species have nearly identical shells, particularly the younger specimens, which makes it very hard to distinguish them (L. Génio personal communication). Therefore, sequences of “*I.*” *simpsoni* from Atlantic and Mediterranean were also obtained from GenBank and added to the my data analysis.

My dissertation aims to study the genetic structure of *Idas* populations among deep-sea chemosynthetic habitats of North East Atlantic and Mediterranean. I expect to clarify the

genetic link between deep-sea populations of *Idas* species, contributing to the understanding of molecular mechanisms that regulate biogeographic distributions and biodiversity dynamics of deep-sea fragmented habitats (Breusing et al., 2015).

The main goals of this dissertation are:

- I. To characterize the population structure of *Idas modiolaeformis* and "*I.*" *simpsoni* found at several deep-sea reduced habitats in the NE Atlantic and in the Mediterranean Sea.
- II. To investigate connectivity among the deep-sea reduced and patchy habitats.

To achieve these goals, I conducted a number of genetic analyses, including:

- Species molecular identification;
- Phylogenetic reconstruction;
- Neutrality test of sequences from each species/population;
- Estimates of site-specific genetic-diversity *per* species;
- Analysis of mitochondrial COI haplotypes (haplotype distribution, frequencies and diversity);
- Estimates of population differentiation and partitioning.

Chapter 3. MATERIAL AND METHODS

3.1. Study area

The dataset analyzed in this study includes specimens collected from three locations in the Atlantic Ocean (Gorringe Bank, Darwin and Meknès mud volcanoes in the Gulf of Cadiz) and GenBank sequences obtained from these three sites and four additional locations (one in the Atlantic and three in the Mediterranean Sea). A brief description of geological and biological features of the seven collection sites (Figure 6), as well as hydrodynamic characteristics of the study region, and estimated linear distances between locations (Table 1) are presented below.

3.1.1. Biogeographic features

Setúbal submarine canyon is located in the Portuguese West margin (Figure 6). Rapid and episodic flushing occurring at submarine canyons are pathways for transport of sediment and organic carbon from shelf to deep-sea depths, enhancing local biodiversity. Gorgonians, sponges, hydroids, anemones, sea urchins, sea stars, oysters and brachiopods are some of the deep-sea fauna found on Setúbal canyon (Weaver et al., 2007). GenBank sequences were obtained from mussels collected after 18 and 28 months on mammal bones there were experimentally deployed at 1000 m depth (Génio et al., 2014).

The Gorringe Bank (Gor), located South West of Portugal (Figure 6), is a vast undersea mountain (length: ~ 180 km; ridge's width: ~ 70 km) raising up 5000 m above seafloor (Banda et al., 1995; Karson et al., 2012; Sallarès et al., 2013; Tortella et al., 1997). The Gettysburg and Ormonde seamounts represent the Gorringe Bank highest points, about 50 m below the sea surface (Karson et al., 2012). These elevated structures act as oceanographic barriers inducing local currents, which cause ocean mixing and tidal energy dissipation (Pitcher et al., 2007; Wessel et al., 2010). As a result of these particular local currents, upwelling can occur around the seamounts, providing nutrients to the deep ocean (Pitcher et al., 2007). Thus, primary production is increased, enhancing the occurrence of important ecological communities and a high biodiversity (Wessel et al., 2010). At the Gorringe Bank it is possible to find sponges, corals, fish, small sessile species in abundance, algae, kelp beds, loggerhead turtle and the bottlenose dolphin (Diário da República, 2015; Karson et al., 2012; OCEANA, 2005). Samples used in this study came

from a wood log opportunistically collected near the Gettysburg seamount (Laming et al., 2015b; Rodrigues et al., 2012).

The Gulf of Cadiz (GoC), located in the North East Atlantic Ocean, was originated by the collision between the European and African plates and the migration of the Betic-Rifean Arc (Hensen et al., 2007; Medialdea et al., 2004; Tortella et al., 1997). The GoC region contains many geological structures associated with fluid escape, such as mud volcanoes, mud-carbonate mounds, pockmarks, faults and diapirs (Medialdea et al., 2009). In the GoC, over 40 mud volcanoes (MV's) exist from the continental shelf to about 3500 m depth (Banda et al., 1995; Hensen et al., 2007; Medialdea et al., 2009; OCEANA, 2005). Biologic communities at GoC comprise the phyla Annelida, Arthropoda, Mollusca, Echinodermata, including some symbiont-hosting species (Cunha et al., 2013a). At the Darwin mud volcano (Figure 6) shells of *Bathymodiolus* ("*Bathymodiolus*" *mauritanicus*), *Neptunea contraria* (Linné) and less abundant *Leptaxinus minutus* were registered by Rodrigues et al. (2013; 2008). The Meknès MV (Figure 6) has extensive fields of coral mounds, where Rodrigues et al. (2008) reported the existence of thyasirids (e.g. *Thyasira granulosa*), empty shells of *N. contraria* and some megafauna. Individuals here studied were collected from CHEMOCOLI colonization devices with pinewood cubes and packed alfafa substrate (Cunha et al., 2013b; Laming et al., 2015b; Rodrigues et al., 2012).

Lacaze-Duthiers (LD) canyon is located in the North West Mediterranean Sea (Figure 6). This canyon belongs to the Gulf of Lions, where several submarine canyons incise the continental slope at depths superior to 1000 meters. At LD canyon, coral community holds a rich fauna of invertebrates, such as sponges, echinoderms, oysters, ascidians, brachiopods and bryozoans. Cephalopods and fishes also occur in LD canyon (Fiala-Medioni et al., 2012). Mussels were recovered from an experimental deployment of palm trunk and pinewood substrate with CHEMOCOLI (Laming et al., 2015b).

Amsterdam mud volcano (Ams) is situated in the Eastern Mediterranean Sea at the Anaximander Mountains, South of Turkey (Figure 6). The Anaximander Mountains are associated to high tectonic deformation and fluids release. Amsterdam MV is characterized by high methane concentrations and extensive turbid fluid expulsion (Charlou et al., 2003). Amsterdam mussel samples were collected from carbonate crusts (Laming et al., 2015b).

The Nile Deep Sea Fan (NDSF) is a sedimentary edifice, located at Eastern Mediterranean Sea (Figure 6). NDSF comprise numerous sub-circular pockmarks (central zone) and several gas-emitting mud volcanoes, with high methane and salinity levels (Dupré et al., 2007, 2014; Foucher et al., 2009). The pockmarks host urchins, bivalves, tubeworms and crustaceans. At NDSF mud volcanoes, chemosynthetic and other deep-sea

fauna was observed, such as polychaetes, bivalves, shrimps, copepods, amphipods, Chaceon crab and fishes (Duperron et al., 2008; Dupré et al., 2007). Individuals DNA sequences used in this study were obtained from mussels found in different substrates: wood, carbonate and alfafa (CHEMOCOLI) (Laming et al., 2015b), authigenic carbonate crusts (Duperron et al., 2008; Lorion et al., 2012), tubes of the siboglinid tubeworms and wood chips (Lorion et al., 2012).

Table 1 – Geographical linear distance matrix (km) between pairs of populations. Values were calculated from coordinates, using an online tool (www.movable-type.co.uk/scripts/latlong.html). *Distances not calculated.

Set	Gor	Dar	Mek	LD	Ams	NDSF	
0	*	*	409	1161	*	2607	Set
	0	420	448	1442	3840	3837	Gor
		0	47	1213	3376	3456	Dar
			0	1237	3374	3450	Mek
				0	2444	2607	LD
					0	311	Ams
						0	NDSF

3.1.2. Hydrodynamic features

The location of the GoC, between the Atlantic and the Mediterranean, its morphology and also biogeochemical features lead to a peculiar hydrology in this area (Macias et al., 2014; Villanueva and Gutiérrez-Mas, 1994). The exchange of water between the Atlantic and the Mediterranean take place at the Gibraltar Strait. The Mediterranean water has elevated levels of salinity, being denser than the Atlantic water. These differences in water salinity and temperature, favour the superficial inflow of Atlantic water and the deep outflow of Mediterranean water (Villanueva and Gutiérrez-Mas, 1994). The water that comes from the Mediterranean forms meddies on the Iberian slope and on the Gorringe Bank (Quentel et al., 2011; Serra and Ambar, 2002).

The Mediterranean outflow water has three layers: the superficial, the central and the deeper. The superficial flow (500-600 m of depth), after passing by Cape San Vicente, takes direction to North, running along the Portugal continental margin. The central flow (700 and 900 m of depth) departs from the continental margin towards the NNW. The deeper flow (1200 and 1500 m of depth) runs to the NW Atlantic, passing between Cape San Vicente and Gettysburg seamount (Villanueva and Gutiérrez-Mas, 1994).

The Gulf of Cadiz slope current and offshore flow are the two main inflows to Mediterranean Sea. The Gulf of Cadiz slope current (inshore flow) comes from Cape S. Vicente to Gibraltar Strait, while the offshore flow comes more southern (Peliz et al., 2009; Villanueva and Gutiérrez-Mas, 1994).

3.2. Sampling collection

Samples of small mussels were collected from wood blocks deployed at Darwin and Meknès mud-volcanoes (Gulf of Cadiz) within the scope of CHEMECO project. Additional mussel samples were obtained from a wood log opportunistically found near the Gettysburg seamount (Gorringe Bank) (Cunha et al., 2013b; Rodrigues et al., 2012). Sampling information is detailed in Table 2. On board, artificial substrates with attached macrofauna were preserved in 96% ethanol. Later, samples were sorted and mussel shells and tissue were separated and placed in new vials (new ethanol solution).

Table 2 – Sampling site details: collection site, respective coordinates (Latitude/ Longitude) and depth (m), cruise and year of sampling (date), number of specimens used for DNA extraction (N).

Site	Latitude/ Longitude	Depth (m)	Cruise (date)	N
Gorringe Bank (Gor)	36°38.557' N 11°36.187'W	1296	Nautilus (2011)	24
Darwin MV (Dar)	35°23.523'N 07°11.513'W	1100	B09/14 (2009)	24
Meknès MV (Mek)	34°59.091'N 07°04.424'W	698	B09/14 (2009)	29

3.3. Molecular analyses

DNA extraction was performed using three different extraction kits: DNeasy Blood & Tissues kit (Qiagen, Valencia, CA), QIAamp DNA Micro kit (Qiagen, Valencia, CA) and ISOLATE II Genomic DNA kit (Bioline, Taunton, MA).

Most of the extracted DNA was 1% diluted for amplification of the mitochondrial DNA Cytochrome Oxidase subunit I (mtCOI). PCR temperature profile was equal for all samples: 94-4' - [95-1' - 47-1' - 72-1'] - 72-10' (x35) (Rodrigues et al., 2012). The primers used were LCO-1560F (5'-ATRCTDATTCGWATTGA-3') and HCO-2148R (5'-CCYCTAGGRTCATAAAAAGA-3') (Jones et al., 2006). Two different PCR protocols were used:

Protocol A - In a total volume of 20.0 μL , 2.0 μL of DNA template (1/100 dilution) and 13.8 μL MilliQ water, 0.5 μL of 10 μM both forward and reverse primers, 0.4 μL of 10 mM dNTP mix (Bioline, UK), 2.0 μL of 10x NH_4 reaction buffer (Bioline, UK), 0.7 μL of 50 mM MgCl_2 solution (Bioline, UK), 0.1 μL of 5 u/ μL BIOTAQTM DNA polymerase (Bioline, UK);

Protocol B - In a total volume of 20 μL , 1.0 μL of DNA template (not diluted), 14.7 μL MilliQ water, 4.0 μL of 5x MyTaqTM reaction Buffer (Bioline, UK), 0.1 μL of 100 μM both primers and 0.1 μL of 5 u/ μL MyTaqTM DNA polymerase (Bioline, UK).

Most of the times, a nested PCR was carried out using the product of the first PCR as DNA template. Thermocyclers used were TProfessional basic or Biometra TRIO (Biometra, Analytik Jena). For more detailed information about molecular methods for each sample see Appendix I – Supplementary Table I.

PCR products were visualized through gel electrophoresis, using the molecular ladder HyperladderTM 50bp (Bioline, UK). Agarose gel was prepared with 0.8 g agarose, 80 mL 1x TAE buffer (Thermo Scientific) and 5 μL Sybersafe (ThermoFisher Scientific) or SyberGreen (NZYTech). Electrophoresis images were obtained using BIO-RAD Molecular Imager[®] ChemiDocTM XRS+.

PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia CA) and sent for bidirectional sequencing at the MacroGen laboratory (Amsterdam).

3.4. GenBank sequences

The accession number, reference and collection site of the sequences retrieved from GenBank are shown in Table 3. Sequences from additional mussel species were added as outgroups for the phylogenetic reconstruction, based on the more recent phylogeny of Bathymodiolins (Lorion et al., 2013). Sequences added were: ESU M (accession number: FJ937202.1), ESU J (FJ937190.1), ESU I (FJ937187.1), *Idas washingtonia* (AY275546.1), *Idas macdonaldi* (AY649804.1), *Adipicola iwaotakii* (EU702322.1), *Adipicola longissima* (FJ937059.1), *Idas japonica* (FJ937078.1), *Benthomodiolus lignicola* (AY275545.1) and *Modiolus modiolus* (U56848.1).

Table 3 - GenBank sequences added to data set. Collection site (site), respective site coordinates (Latitude/ Longitude) and depth (m), number of sequences (N), accession numbers, reference (R). a) Laming et al., 2015b; b) Rodrigues et al., 2012; c) Gaudron et al., 2010; d) Duperron et al., 2008; e) Lorion et al., 2012; f) Génio et al., 2014.

Spp	Site	Latitude/ Longitude	Depth (m)	N	Accession number	R
<i>Idas modiolaeformis</i>	Gorringe Bank (Gor)	36°39'N 11°36'W	1296	3	KT216487.1, KT216488.1 HE964759.1	a) b)
	Darwin MV (Dar)	35°24'N 07°12'W	1100	4	KT216482.1 - KT216484.1 HE964757.1	a) b)
	Meknès MV (Mek)	34°59'N 07°04'W	698	3	KT216485.1, KT216486.1 HE964758.1	a) b)
	Lacaze-Duthiers Canyon (LD)	42°33'N 3°25'E	525	3	KT216497.1, KT216499.1, KT216500.1	a)
	Amsterdam MV (Ams)	35°20'N 30°16'E	2031	2	KT216492.1, KT216493.1	a)
	Nile Deep-Sea Fan (NDSF)	32°32'N 30°21'E; 32°38'N 29°55'E; 32°38'N 29°55'E; 32°22'N 31°42'E; 32°32'N 30°21'E; 32°08'N 28°09'E	1693 2129 1150- 3000	25	FM212787.1 EF210072.1 FJ158565.1 - FJ158587.1	c) d) e)
<i>“Idas” simpsoni</i>	Setúbal Canyon (Set)	38°17'N 09°07'W	1000	32	KT216489.1- KT216491.1 HG931851.1 - HG931879.1	a) f)
	Lacaze-Duthiers Canyon (LD)	42°33'N 3°25'E	525	3	KT216498.1, KT216501.1, KT216502.1	a)

Figure 6 shows the total number of individuals used in this analysis for each species *per* location. *Idas modiolaeformis* sequences originated from 6 locations: Gorringe Bank (Gor), Darwin MV (Dar), Meknès MV (Mek), Lacaze-Duthiers canyon (LD), Amsterdam MV (Ams) and Nile Deep-Sea Fan (NDSF). The first three locations occur in the East Atlantic, and the last three occur in the Mediterranean. Sequences from “*Idas*” *simpsoni* were from 3 locations: Setúbal canyon (Set), Meknès MV – East Atlantic; and Lacaze-Duthiers canyon – Mediterranean.

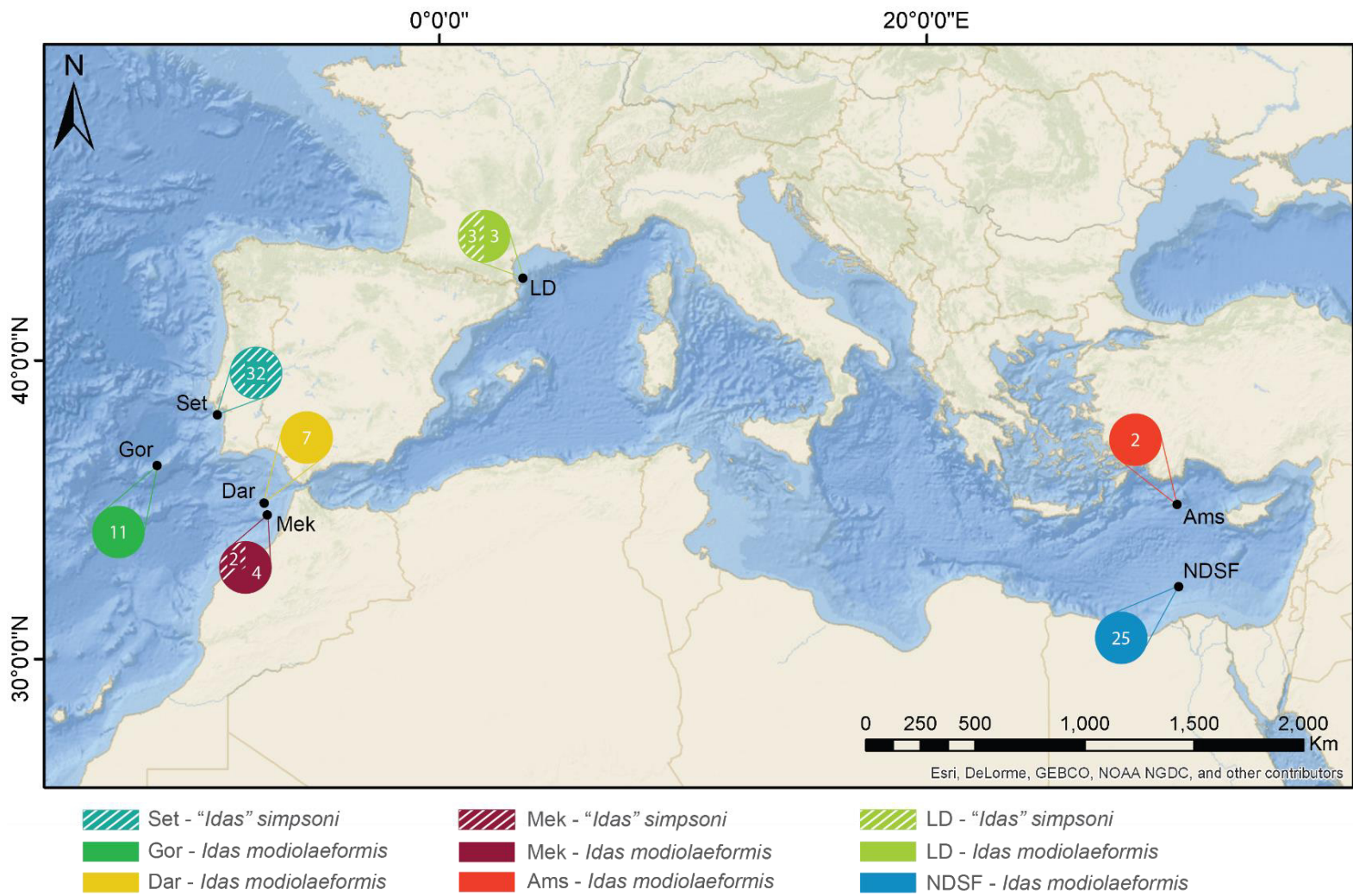


Figure 6 –Localization of the study areas (populations) and the respective number of individuals (sequences) in this study for each population. Populations are represented in different colors. Sequences of "*I.*" *simpsoni* are represented with lines and *I. modiolaeformis* without lines. Map by Mariana Morgado. Pie charts by Jorge Malafaia.

3.5. Data analyses

A total of 93 sequences were analysed, corresponding to 15 sequences obtained in this study and 78 sequences acquired in GenBank (Benson et al., 2005).

All 15 sequences obtained in this study were trimmed, paired and clipped using BioEdit Sequence Alignment Editor version 7.2.5 (Hall, 1999). Gaps were filled manually, as well as conservative sites to increase sequence length. The total length of all the sequences (obtained in this study and from the GenBank) obtained was 437bp. To ensure the correct translation of the sequence from nucleotide to protein, sequences were evaluated using EMBOSS online tool Transeq (Goujon et al., 2010; Rice et al., 2000) (available from www.ebi.ac.uk/Tools/st/emboss_transeq). All sequences were aligned using ClustalW Multiple alignment with 1000 bootstraps (NJ tree) run in BioEdit software. After the alignment, smaller sequences were excluded (4), obtaining a total of 89 sequences (14 sequences of this study and 75 sequences of GenBank).

Best Fit Model was run in MEGA 6.0 (Tamura et al., 2013). Phylogenetic maximum likelihood (ML) tree was performed using MEGA 6.0 software, selecting 1000 bootstraps, Tamura and Nei (1993) nucleotide substitutions model (determined by Best Fit Model), gamma distribution for rate variation among polymorphic sites (5 discrete gamma categories), first and second codon positions and Nearest-Neighbour-Interchange (NNI) for inference options of ML heuristic method.

DnaSP software v. 5.10.01 (Rozas, 2009) was used to calculate several genetic diversity indices: number of sequences; number of polymorphic/segregating sites (S); total number of mutations (Eta); number of haplotypes (h); haplotype diversity (Hd) with respective variation and standard deviation and nucleotide diversity (π). Tajima's and Fu and Li tests were performed with DnaSP to validate data neutrality.

Haplotype network was obtained with Network 4.6.1.4 (Fluxus Technology Ltd, 1999-2016), using a median-joining method (Bandelt et al, 1999). ARLEQUIN software v. 3.5.2.2 (Excoffier and Lischer, 2010) was used to assess genetic structure through AMOVA ($k=1$). Significance tests were calculated with 1023 permutations. Spatial Analysis of Molecular Variance (SAMOVA) is an AMOVA that correlates geographical and genetic distances among populations. This analysis was performed for different numbers of groups ($k=2, 3, 4$ and 5) with SAMOVA 1.0 (Dupanloup et al., 2002). Pairwise genetic distances were calculated in ARLEQUIN and plotted against geographical distances showed in Table 1 (Microsoft Excel 2016). Mantel test for matrix correlation between genetic (F_{st}) and geographic (km) distances was performed with 1000 permutations using the online Isolation

By Distance Web Service (IBDWS) version 3.23 (genetic distances/ similarities):
<http://ibdws.sdsu.edu/> (Jensen et al., 2005). Negative genetic distances were not included.

Chapter 4. RESULTS

4.1. Sequencing success rate

DNA was extracted from a total of 77 mussel individuals (Table 2), but it was only possible to amplify and sequence the mtCOI gene of 15 specimens (Appendix I). Less than half of the extracted samples were successfully amplified (43%), and the number of successful sequences obtained was then reduced to 42%, giving a final success rate of 18% (excluding one partial sequence) (Figure 7).

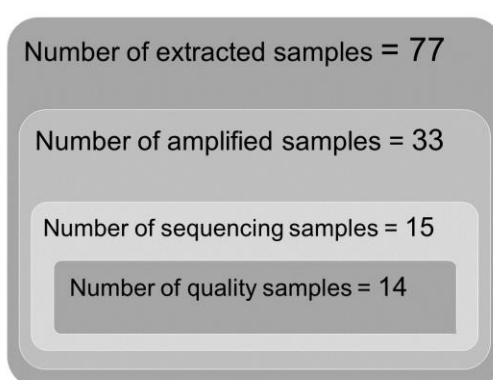


Figure 7 – Number of samples obtained after each laboratory step.

Out of the 14 sequences obtained, 12 sequences belong to the species *Idas modiolaeformis* and two belong to “*Idas*” *simpsoni*. Overall, it was possible to sequence eight specimens of *I. modiolaeformis* from Gorrington Bank, three individuals from Meknès MV (one *I. modiolaeformis* and two “*I.*” *simpsoni*) and three individuals of *I. modiolaeformis* from Darwin MV (Table 4).

Table 4 – Number of sequences obtained for each species *per* location.

	Gorrington Bank	Darwin MV	Meknès MV	Total
<i>Idas modiolaeformis</i>	8	3	1	12
“ <i>Idas</i> ” <i>simpsoni</i>	0	0	2	2
Total	8	3	3	14

4.2. Phylogenetic reconstruction

Overall, sequences from this study and from GenBank comprise 52 sequences of *Idas modiolaeformis* and 37 sequences of “*Idas*” *simpsoni*, from 7 locations in the North East Atlantic and Mediterranean Sea.

The phylogenetic tree clearly reveals two main groups that separate each studied species into one of two different clades (Figure 8). Within each species, no segregation was observed for the different geographic locations.

Tajima's D test did not show statistical significance for each singular population and for all populations of *Idas modiolaeformis* (Table 5), meaning that these sequences follow a neutral evolution model. The Lacaze-Duthiers canyon (LD) population is not shown in Table 5, because Tajima's D test requires a minimum of 4 sequences to perform calculations.

The Tajima's D value obtained for "*Idas*" *simpsoni* showed statistical significance for Setúbal population and for all populations (Table 5), which means that mtCOI gene of this species is not in accordance with neutral evolution. The number of sequences from Meknès (N=2) and LD (N=3) was too low to perform Tajima's D test.

Fu and Li tests for neutrality were performed too, revealing the same results as Tajima's D test (see in Appendix II – Supplementary Table II).

Table 5 – Results of Tajima's test for neutrality. Ns means not significant.¹ All individuals of *I. modiolaeformis* including sites in Table plus LD.² All individuals of "*I.*" *simpsoni* from Setúbal, LD and Meknès.

<i>Idas modiolaeformis</i>		
Populations	Tajima's D	Statistical significance
Gor	0.798	Ns, P > 0.10
Dar	-0.813	Ns, P > 0.10
Mek	-0.847	Ns, P > 0.10
NDSF	-1.108	Ns, P > 0.10
All ¹	-0.999	Ns, P > 0.10
" <i>Idas</i> " <i>simpsoni</i>		
Populations	Tajima's D	Statistical significance
Set	-1.982	*, P < 0.05
All ²	-2.132	*, P < 0.05

4.3. Site-specific genetic-diversity *per species*

A summary of site-specific genetic diversity for each species is shown in Table 6. All sequences of Atlantic populations had the highest number of polymorphic sites, mutations and haplotypes. Excluding populations with low number of sequences (N<5), haplotype diversity for both species is higher for Atlantic populations.

The *Idas modiolaeformis* NDSF population was best represented, with 25 sequences. The remaining populations had less than 15 sequences. LD population for *I. modiolaeformis* had no polymorphic sites, corresponding to a single haplotype. Due to the low number of sequences (two) analysed, corresponding to two different haplotypes, Amsterdam MV had

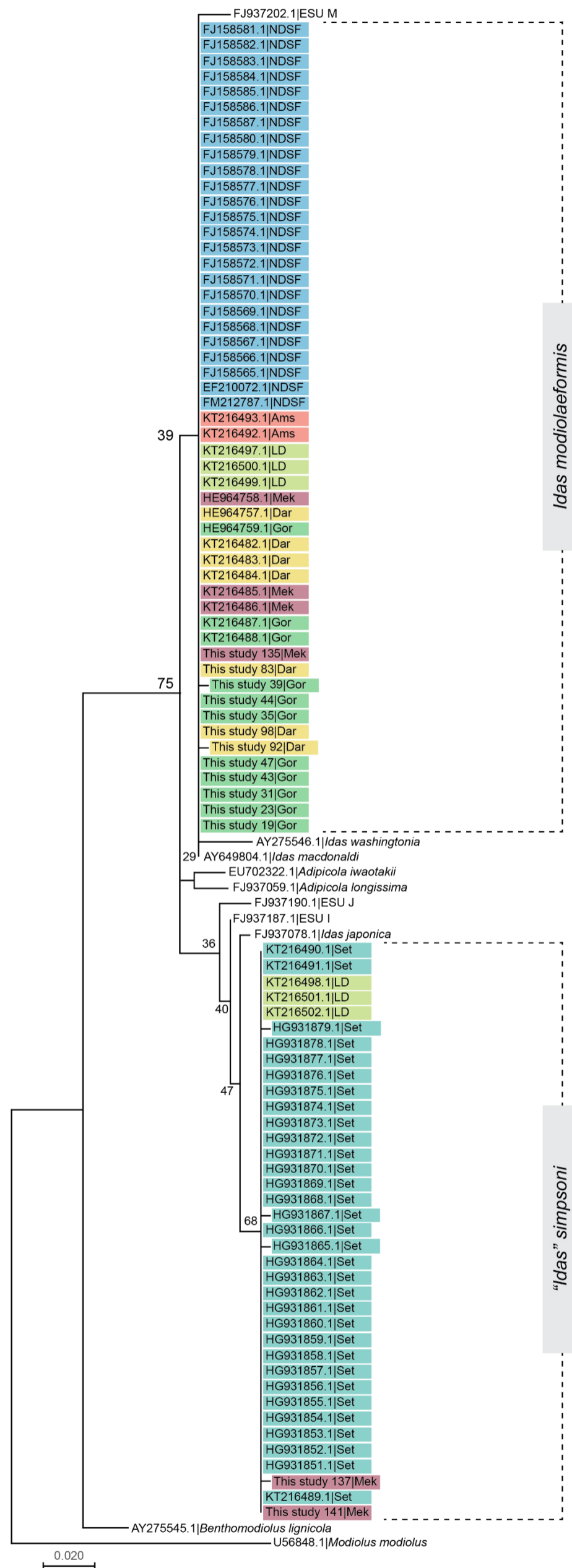


Figure 8 – Phylogenetic ML tree, nucleotide substitutions model Tamura-Nei 1993 with 1000 bootstrap and 5 discrete gamma categories for rate variation among polymorphic sites. Bootstrap support values are shown next to the branches. Scale bar represents estimated sequence divergence. Samples are identified by accession number and respective location name abbreviation. Colors indicate populations. Set – Setúbal Canyon, Gor – Gorringe Bank, Dar – Darwin MV, Mek – Meknès MV, LD - Lacaze-Duthiers Canyon, Ams – Amsterdam MV, NDSF – Nile Deep-Sea Fan.

the highest haplotype diversity, but this value is not meaningful. The second highest haplotype diversity value was found in Darwin MV, with seven sequences analysed. The NDSF population had the smallest haplotype diversity, with 25 sequences analysed. Meknès sequences had the highest nucleotide diversity, once the number of mutations *per* sequence was higher. NDSF sequences had the lowest nucleotide diversity. All 52 sequences of *I. modiolaeformis* revealed 11 haplotypes, generated by 26 mutations.

The most represented population of "*I.* *simpsoni*" was Setúbal canyon (32 sequences). The remaining populations had less than five sequences, preventing further statistical analysis. Setúbal canyon had the highest number of segregating sites and mutations. Meknès MV sequences only had one mutation. Haplotype diversity reached the maximum for Meknès and LD, but again these values are not meaningful because of a low number of sequences *per* population. Nevertheless, Setúbal canyon had a high haplotype diversity, with 32 sequences analysed. The nucleotide diversity was higher for LD sequences and smaller for Meknès sequences. From the total of 37 sequences of "*I.* *simpsoni*", 18 mutations (in 17 polymorphic sites) originated 16 haplotypes.

Table 6 – Site-specific genetic-diversity *per* species for both species. Atlantic (Atlan.) and Mediterranean (Med.) regions, include some populations. Populations refer to sample site. N - number of sequences used for each population, S - number of polymorphic sites, Eta - number of mutations, h - number of haplotypes, Hd - haplotype diversity, Var Hd - variance of haplotype diversity, SD Hd - standard deviation of haplotype diversity, $\pi \times 100$ - nucleotide diversity.

<i>Idas modiolaeformis</i>									
Regions	Populations	N	S	Eta	h	Hd	Var Hd	SD Hd	$\pi \times 100$
Atlan.	Gor	11	18	18	5	0.618	0.027	0.164	1.656
	Dar	7	21	21	4	0.714	0.033	0.181	1.678
	Mek	4	15	15	2	0.500	0.070	0.265	1.716
Med.	LD	3	0	0	1	0	0	0	0
	Ams	2	1	1	2	1.000	0.250	0.500	0.229
	NDSF	25	3	3	4	0.410	0.012	0.111	0.101
	All	52	26	26	11	0.689	0.004	0.065	0.914
<i>"Idas" simpsoni</i>									
Regions	Populations	N	S	Eta	h	Hd	Var Hd	SD Hd	$\pi \times 100$
Atlan.	Set	32	14	15	13	0.770	0.005	0.074	0.344
Med.	Mek	2	1	1	2	1.000	0.250	0.500	0.229
	LD	3	3	3	3	1.000	0.074	0.272	0.458
	All	37	17	18	16	0.782	0.005	0.068	0.351

4.4. Population structure

Idas modiolaeformis and “*I.*” *simpsoni* were clearly segregated by 71 mutations. *Idas modiolaeformis* sequences were distributed by 11 haplotypes (six from Atlantic, three from Mediterranean, two shared between the two regions), and “*I.*” *simpsoni* had 16 haplotypes (13 from Atlantic, two from Mediterranean, one shared) (Table 7 and Figure 9).

Haplotype 1 was the most frequent haplotype within *I. modiolaeformis* species (N=28 – Table 7), shared by individuals from Atlantic (Gorringe and Darwin) and Mediterranean (NDSF and Amsterdam). Haplotype 2 gathered individuals from NDSF and Amsterdam populations that are both in the Mediterranean. Meknès and LD populations that are geographically separated by Gibraltar Strait shared Haplotype 7. Haplotypes 9, 10 and 11 revealed higher divergences from the remaining haplotypes and were found only in Atlantic populations (Gorringe, Darwin and Meknès).

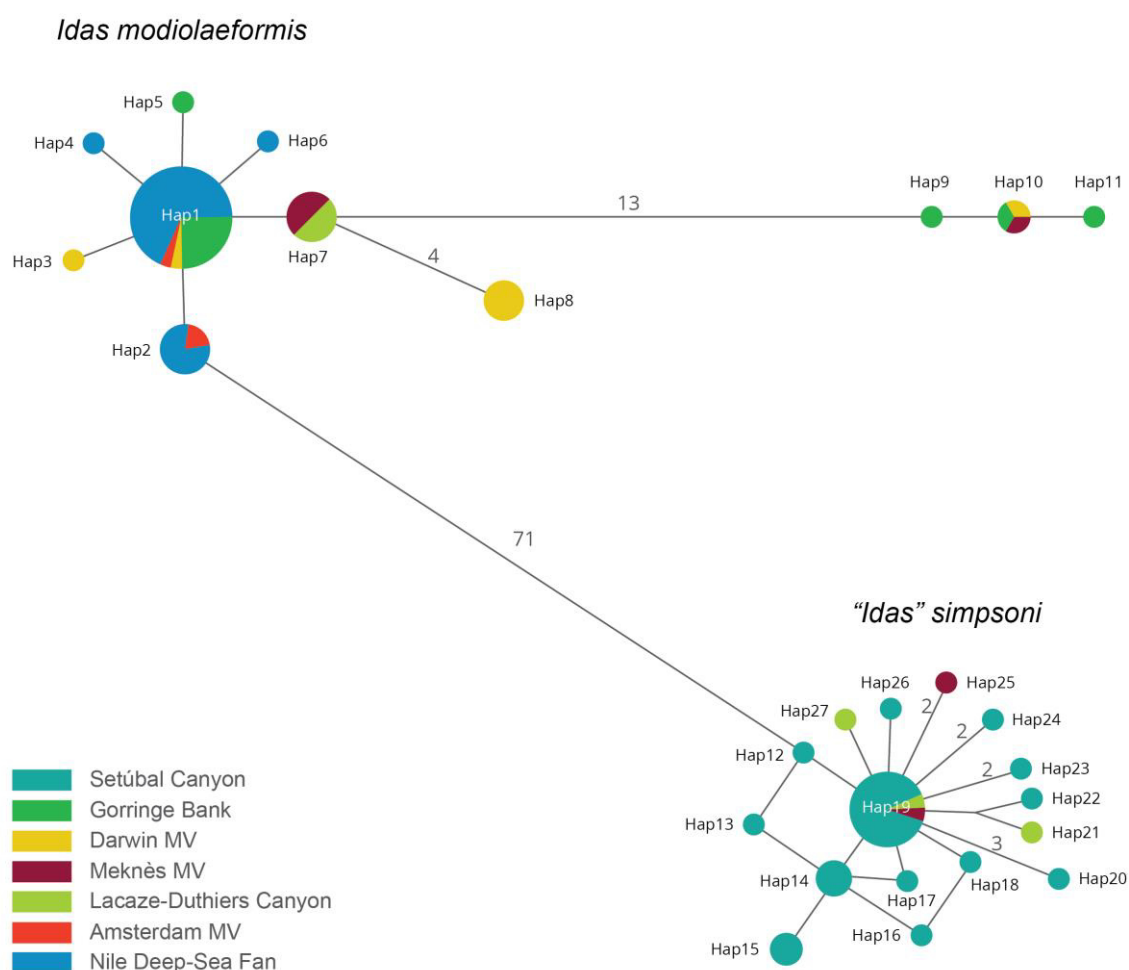


Figure 9 – Median-joining haplotype network for mtCOI sequences of *Idas modiolaeformis* e “*Idas*” *simpsoni*, from 7 populations. Lines represent number of mutations. All haplotypes differ by one mutation; exceptions are referred with the respective number of mutations. Circle size is proportional to the number of individuals each haplotype contains.

All populations of “*Idas*” *simpsoni* had one haplotype in common, Hap19 (n=17–Table 7). Populations from Setúbal and LD canyons had two very close haplotypes, with only one mutation segregating them (Hap21 and Hap22 – Figure 9).

In both species Meknès and LD populations shared one haplotype (Hap7 and Hap19, respectively in *I. modiolaeformis* e “*I.*” *simpsoni*).

Table 7 – Number of individuals *per* haplotype and population. Haplo. – Haplotype, Pop – Population, N. of ind./pop – number of individuals *per* population, N. of ind./hap – number of individuals *per* haplotype.

<i>Idas modiolaeformis</i> haplotypes				“ <i>Idas</i> ” <i>simpsoni</i> haplotypes			
Haplo.	Pop	N. of ind./pop	N. of ind./hap	Haplo.	Pop	N. of ind./pop	N. of ind./hap
Hap1	NDSF	19	28	Hap12	Set	1	1
	Dar	1		Hap13	Set	1	1
	Ams	1		Hap14	Set	4	4
	Gor	7		Hap15	Set	3	3
Hap2	NDSF	4	5	Hap16	Set	1	1
	Ams	1		Hap17	Set	1	1
Hap3	Dar	1	1	Hap18	Set	1	1
Hap4	NDSF	1	1	Hap19	Set	15	17
Hap5	Gor	1	1		Mek	1	
Hap6	NDSF	1	1		LD	1	
Hap7	Mek	3	6	Hap20	Set	1	1
	LD	3		Hap21	LD	1	1
Hap8	Dar	4	4	Hap22	Set	1	1
Hap9	Gor	1	1	Hap23	Set	1	1
Hap10	Gor	1	3	Hap24	Set	1	1
	Dar	1		Hap25	Mek	1	1
	Mek	1		Hap26	Set	1	1
Hap11	Gor	1	1	Hap27	LD	1	1

A very great genetic differentiation ($F_{st}=0.35>0.25$, $p\text{-value}=0$) was observed among all *I. modiolaeformis* populations in the Atlantic and Mediterranean, indicating that these populations are structured. Most of the variation (64.5%) is explained by differences within populations (Table 8).

Table 8 – AMOVA (k=1) for *Idas modiolaeformis*. Source of variation, d.f., variance components (Var Comp) and percentage of variation. *** p<0.001.

Source of variation	d.f.	Sum of squares	Var Comp	% of variation
Among populations	5	6.173	0.136 Va	35.50
Within populations	46	11.404	0.248 Vc	64.50
Total	51	17.577	0.384	
Fixation Index		Fst: 0.355	p-value: 0.000±0.000***	

According to SAMOVA results (Table 9), it was possible to consider two hypotheses for population structure partitioning, namely in 3 or 4 groups (k=3; k=4). In both scenarios, Fct values indicated great genetic differentiation (Fct>0.25) among groups and the respective p-value had statistical significance. The three-group scenario aggregates 1) LD, Amsterdam and NDSF; 2) Darwin and 3) Gorringe and Meknès. Four-group scenario corresponds to 1) Amsterdam and NDSF; 2) Darwin; 3) Gorringe and 4) Meknès and LD.

Table 9 – SAMOVA (k=2,3,4,5) for *Idas modiolaeformis*, obtained with SAMOVA software. Number of groups (k), fixation indexes: Fct (among groups), Fsc (among populations within groups) and Fst (within populations). *p<0.05 **p<0.01.

k	Group composition	Fct	P-value	Fsc	P-value	Fst	P-value
2	1. Gor + Mek + LD + Ams + NDSF 2. Dar	0.175	0.180	0.183	0.012*	0.327	0.010**
3	1. LD + Ams + NDSF 2. Dar 3. Gor + Mek	0.286	0.017*	- 0.011	0.140	0.278	0.013*
4	1. Ams + NDSF 2. Dar 3. Gor 4. Mek + LD	0.347	0.029*	- 0.141	0.319	0.255	0.013*
5	1. Ams + NDSF 2. Dar 3. Gor 4. Mek 5. LD	0.429	0.075	- 0.305	0.295	0.255	0.011**

Among *I. modiolaeformis* populations (Table 10), a significant p-value for the estimated pairwise genetic distance (Fst) was detected between all Atlantic populations (Gorringe and Darwin, Gorringe and Meknès, Darwin and Meknès), and also between Atlantic and some Mediterranean populations (Gorringe and LD, Darwin and LD, Darwin and NDSF, Meknès and NDSF), and finally between two populations in either side of Mediterranean (LD and

NDSF). Significant p-values indicate significant distances between two populations. All remaining pairs of populations showed no significant p-values, meaning that no significant genetic divergences were detected between them.

Table 10 – Pairwise genetic distances of *Idas modiolaeformis* populations. Fst values below diagonal and p-values above diagonal. Bold values are significant (p-value<0.05).

	Gor	Dar	Mek	LD	Ams	NDSF
Gor	*	0.012±0.003	0.003±0.002	0.000±0.000	0.685±0.014	0.271±0.0163
Dar	0.262	*	0.008±0.003	0.008±0.003	0.338±0.016	0.000±0.000
Mek	0.408	0.349	*	0.999±0.000	0.180±0.010	0.000±0.000
LD	0.542	0.514	-0.091	*	0.111±0.010	0.000±0.000
Ams	-0.069	0.147	0.344	0.647	*	0.473±0.015
NDSF	0.016	0.423	0.569	0.659	-0.058	*

In order to find the best-fit genetic structure model for *I. modiolaeformis* populations, geographical distances (km) were plotted against genetic distances (pairwise Fst values) (Figure 10). Negative Fst values were excluded. Trendline R-square is very low, indicating that geographic and genetic distances are not correlated, as also confirmed by Mantel test ($r < 0$, $p = 0.45$ and $r > 0$, $p = 0.55$).

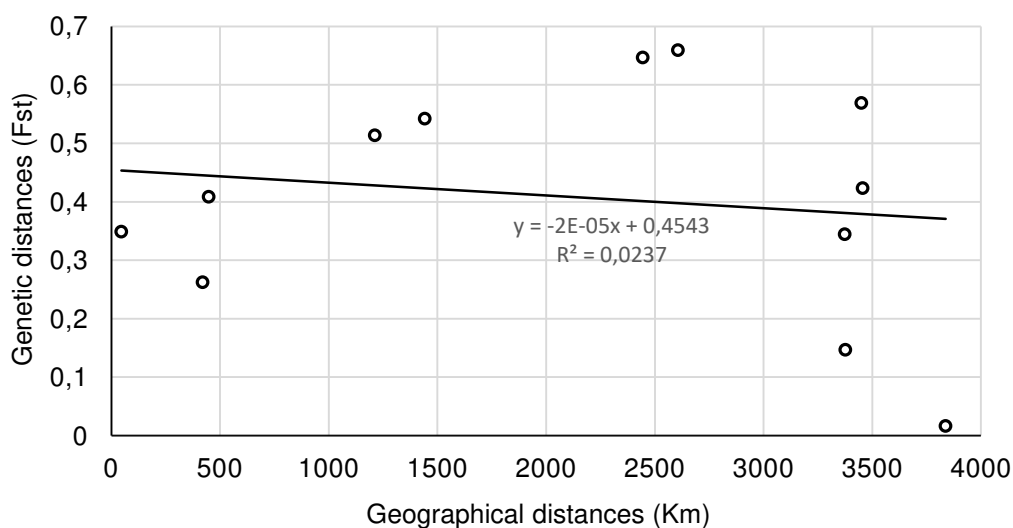


Figure 10 – Relationships between geographical linear distances and pairwise genetic distances (Fst) of *I. modiolaeformis* populations.

Chapter 5. DISCUSSION

5.1. Sequencing success rate

In this study, the success of molecular sequencing was very low (18%). Other studies revealed unsuccessful amplification of mitochondrial genes, NADH dehydrogenase subunit 4 in mussels (Boschen et al., 2015; Faure et al., 2015) and mtCOI in crustaceans (Asmyhr and Cooper, 2012). The amplification success rate of Asmyhr and Cooper (2012) was less than 20%, a lower value than the present study amplification rate (43%).

One possible explanation for the low success rate is related to the preservation of specimens. Recommendations indicate any fixative to have larger volume (5x-10x) than the specimen volume (Geiger et al., 2007). Because the specimens were preserved still attached to the wood cubes (CHEMOCOLI samples) and wood log (Gorringe samples) the volume of ethanol should have been much higher than the one used. It is also possible that ethanol did not reach the tissue inside the shells. Concerning fixation of small mussels for molecular work, it is recommended to drill or break the shell, so that the ethanol reaches the tissue. In order to preserve the shell for taxonomic identifications and other envisioned approaches, shell drilling was not performed. Regarding the storage of the tissue it is advised to replace the ethanol solution in the first days, due to tissue dehydration (Geiger et al., 2007; Prendini et al., 2002). The shell and tissue of most samples were later (> one year) separated and kept in 96% ethanol, with replacement of the solution, although some tissue dehydration could have happened during specimen dissection.

5.2. Phylogenetic reconstruction

The phylogenetic tree showed a clear separation of *I. modiolaeformis* and "*I.*" *simpsoni*, and no segregation of individuals within each species *per* geographic location. The tree branches had low support because only the mitochondrial gene was used, and a combination of this and more conservative genes (e.g. nuclear markers) is needed to resolve phylogenetic relationships (Hartl and Clark, 1997).

Regarding neutral evolution of the mtCOI gene, results revealed neutrality for *I. modiolaeformis*, but not for "*I.*" *simpsoni*. Thus, divergence results for *I. modiolaeformis* can be interpreted as a balance of gene flow (when divergence is reduced) and genetic drift (when divergence is increased). In the case of "*I.*" *simpsoni* neutrality test was probably biased due to the low number of sequences *per* population (Mek: N=2; LD: N=3 – Table 7). Nevertheless, Setúbal population had a good representation (N=32) and still Tajima's D test

did not support neutrality for mtCOI gene, suggesting that this gene might be under selection. Therefore, gene flow and genetic drift cannot be considered the only factors responsible for the genetic differences, as natural selection has to be considered as well (Allendorf and Luikart, 2007; Hartl and Clark, 1997). For these reasons, genetic differentiation analyses (AMOVA, SAMOVA, Fst pairwise distances) were not performed for “*I.*” *simpsoni*.

5.3. Site-specific genetic-diversity *per species*

A great discrepancy of the number of sequences analyzed *per* population in each species was translated also in great differences in estimated genetic indices. A low number of individuals *per* site (<10-20) can either underestimate or overestimate indices, thus all interpretations have to be made with caution. For instance, maximum haplotype diversity ($H_d=1$) was found when the number of sequences was low (2-3) and each one represented a different haplotype (e.g. Amsterdam population of *I. modiolaeformis*; Meknès and LD populations of “*I.*” *simpsoni*).

Nevertheless, genetic diversity values reported in this study (Table 6) are within the range of observed values in other bathymodiolin species. The values of polymorphic sites (S) in *I. modiolaeformis* and “*I.*” *simpsoni* were 26 and 17, respectively. Faure et al. (2015) reported 21 polymorphic sites in *Bathymodiolus brooksi*, while in *B. thermophilus* varied between 8 and 27 (Plouviez et al., 2013). I found a haplotype diversity of 0.689 for *I. modiolaeformis* and 0.782 for “*I.*” *simpsoni*, fitting the values already observed in other deep-sea mussels. Haplotype diversity ranged between 0.292 (*B. nov. sp. GoM*) to 0.909 (*B. childressi*) in Faure et al. (2015) study.

Comparing Atlantic and Mediterranean populations for both species, Atlantic populations showed higher values of polymorphic sites, mutations and haplotypes indicating a higher genetic diversity. It is interesting to note that Darwin population for *I. modiolaeformis*, with only seven individuals, showed a high value of polymorphic sites ($S=21$) and haplotype diversity ($H_d=0.714$) compared to NDSF population that showed a lower number of polymorphic sites and genetic diversity ($S=3$; $H_d=0.410$) within 25 individuals sampled. Although sampling limitations can influence haplotype diversity, these results may suggest that NDSF population is more isolated. Overall, “*I.*” *simpsoni* exhibited 16 haplotypes out of 37 individuals, while *I. modiolaeformis* with more individuals (52) showed only 11 haplotypes. Numerous haplotypes further support the rejection of gene neutrality, suggesting that “*I.*” *simpsoni* population is under selection.

5.4. Population structure

Idas modiolaeformis and “*I.*” *simpsoni* are separated by 71 mutations, as shown in the haplotype network (Figure 9). The occurrence of one dominant haplotype for each species (Hap1 in *I. modiolaeformis*, and Hap19 in “*I.*” *simpsoni*) suggests ancestral polymorphisms shared between most sampled populations: Dar, Gor, Ams, NDSF for *I. modiolaeformis*, and Set, Mek, LD for “*I.*” *simpsoni*. High genetic differentiation ($F_{st}=0.35$, Table 8) among *I. modiolaeformis* populations across the entire geographic region provides evidence for segregation of populations. Differentiation within populations is responsible for 64.5% of the variation found, in accordance with the highly discrepant site-specific genetic diversity.

Haplotype distribution provides evidence to explain two possible population-partitioning scenarios of *Idas modiolaeformis*. On one hand (scenario $k=3$), the Mediterranean populations (NDSF, Ams and LD) are separated from the Atlantic populations, which are split in two clusters: 1) Gor + Mek and 2) Dar. Darwin population holds two unique haplotypes (Hap3 and Hap8) in 5 out of 7 individuals and shares one more divergent haplotype (Hap10) with the other two Atlantic populations. On the other hand (scenario $k=4$), the two Eastern Mediterranean populations (NDSF and Ams) are segregated from a central group of two populations on both sides of the Gibraltar Strait (LD and Mek), and two other Atlantic populations, Darwin MV and Gorringer Bank. LD unique haplotype (Hap7) is only shared with Meknès, while Gorringer holds three unique haplotypes (Hap5, Hap9 and Hap11).

Although the first partitioning scenario ($k=3$) suggested an apparent segregation between Atlantic and Mediterranean populations, the present data does not allow us to exclude the occurrence of gene flow across the Gibraltar Strait. Connectivity between Atlantic and Mediterranean populations has previously been studied. Affinito et al. (2015) showed high genetic connectivity and bidirectional migration of tunicates between Atlantic and Mediterranean. Correia et al. (2012) also showed genetic connectivity of conger eel between the two areas.

Despite the shorter geographic distances (47-448 km) among Atlantic populations, these sites showed significant ($p\text{-value}<0.05$) pairwise genetic distances (F_{st} : Dar-Gor=0.26, Dar-Mek=0.35 and Mek-Gor=0.41) just slightly lower than populations that are separated by more than 1000 km (F_{st} : Dar-LD=0.51, Gor-LD=0.54 and LD-NDSF=0.66) or even farther away (>3000 km, F_{st} : Dar-NDSF=0.42 and Mek-NDSF=0.57). The lowest (non-significant) pairwise genetic distances were found between populations of two very distant geographic sites (>3300 m), suggesting that sufficient gene flow may be occurring between them. Gene flow between Dar-Ams ($F_{st}=0.15<0.20$) is sufficient to avoid harmful effects of local

inbreeding, whereas higher gene flow between Gor-NDSF ($F_{st}=0.016<0.020$) is sufficient to maintain similar allele frequencies (Lowe and Allendorf, 2010).

The lack of correlation ($R^2=0.024$, $p>0.05$) between geographic and genetic distances (Figure 10) provides evidence for an *island* model of population structure. The low genetic distances could result from the mixing of larvae by ocean currents and their transport over long distances, that may form one large and common gene pool contributing to gene input in smaller populations (Altukhov, 2006). A low genetic differentiation across large geographic regions was previously reported in mussel populations in the East and West Pacific hydrothermal vents (Breusing et al., 2015; Johnson et al., 2013). Moreover, the connection between populations can also be guaranteed by multiple and patchy intermediate habitats that allow gene exchange between closer populations, linking populations that are farther apart (*stepping-stone* model) (Allendorf and Luikart, 2007; Breusing et al., 2015).

Connectivity between Atlantic and Mediterranean mussel populations may be explained by their long-distance dispersal capabilities and ocean currents in this geographic region. *Idas modiolaeformis* larvae are likely planktotrophic and have an estimated pelagic duration of 4-5 months (Gaudron et al., 2012). Similar to other bathymodiolin mussels, it is likely that these larvae perform vertical migration, moving up hundreds of meters to use faster currents at the surface allowing them colonize distant habitats (Arellano et al., 2014). This dispersal strategy can explain the low genetic distances among geographical distant populations (Carney et al., 2006). Superficial inflow of Atlantic water at ~100 m depth (Villanueva and Gutiérrez-Mas, 1994) may transport larvae between regions in the Atlantic to the Mediterranean. However, larval transport may also occur in Mediterranean-Atlantic direction, through Mediterranean outflow water (Villanueva and Gutiérrez-Mas, 1994), considering that larvae would stay in deeper water layers (approx. 500-1500 m of depth). Other examples in literature support the idea that Atlantic populations may serve as larval sources to Mediterranean populations (Bouchet and Taviani, 1992). The higher genetic diversity found in the Atlantic mussel populations suggested that larval transport is more likely to occur from Atlantic to Mediterranean.

Panmictic populations are generally assumed for long-distance dispersal larvae (Arellano and Young, 2009). However, the present data does not support the occurrence of panmixia across the entire geographic region. Yet, sampling gaps can influence the power of differentiation analyses. Our F_{st} values might be overestimated, due to the low number of sequences and a single gene marker (Vrijenhoek, 2010). Further investigations with increased number of sequences (individuals) and populations, and additional genetic data

(e.g., nuclear SNPs and allozyme) are needed to confirm these results. This information will be crucial to understand source-sink dynamics that are useful to conservation management, particularly regarding the inter-MPA connectivity (Bell, 2008). The implementation of the Gorringe Bank MPA demands further connectivity studies in the Atlantic and Mediterranean region for its sustainable management. For instance, it is important to investigate whether deep-sea mussel populations from Atlantic and Mediterranean are connected by contemporary dispersal, or if historic connectivity has persisted to present day and populations did not have enough time to become isolated.

Chapter 6. CONCLUSIONS AND FUTURE RESEARCH

The low number of individuals *per* population as well the discrepant number of sequences among populations limited the interpretation of the results. Nevertheless, the present data indicated that “*Idas*” *simpsoni* and *Idas modiolaeformis* populations from Mediterranean and North East Atlantic are not geographically isolated. The two studied species showed one dominant haplotype suggesting shared ancestral polymorphisms between populations across entire geographic region. In the case of *I. modiolaeformis*, the overall high genetic differentiation indicates that metapopulation is structured, particularly through differences within populations. The low genetic distances found between Atlantic and East Mediterranean populations indicate gene flow over >3000 km distance. Genetic and geographic distances are not correlated, supporting an *island* model of population structure. Additional samples and other genetic markers are needed to clarify the genetic structure of *Idas* metapopulations in the studied region.

This study provided insights into gene exchange between deep-sea habitats in the Mediterranean, Gulf of Cadiz and the Gorringe Bank, which was recently established as a Marine Protected Area. Further studies are necessary to understand the recolonization potential and source-sink dynamics among these areas (Boschen et al., 2015; Marshall et al., 2009), in order to comprehend resilience of populations to natural and human impacts, enabling a sustainable management of deep-sea ecosystems.

In addition to genetic data, demographic studies (size of population, number of self-recruits and migrants, mortality and birth rates) may allow to infer more realistic estimates of population structure. Considering connectivity of organisms with pelagic larvae stage, larval dispersal and hydrology play a relevant role. Studies about timing and location of larval release, larval behavior, mortality rate, PLD estimates and transport distances, are important to better understand dispersal process. Artificial markers (for example, fluorescent dyes) have been used to follow the movement of larvae, nevertheless dilution of larvae in natural environment is very high, making this method unfeasible (reviewed in Levin, 2006). However, natural markers such stable isotope signatures of tissues and geochemical fingerprinting can also be informative about larval dispersal (Cowen and Sponaugle, 2009; Génio et al., 2015; Levin, 2006). Biophysical models incorporating hydrology, three-dimension reality and more realistic estimates of larval biology (behavior, mortality, PLD, etc) are another powerful method to obtain comprehensive information about larval dispersal at various spatial and temporal scales.

Studying deep-sea ecology has financial and technical limitations, so is important gather as much information from the data already collected and share the information to the scientific community, not only through scientific publications but also in online databases. Thus, perspectives of several study areas help to provide more realistic approaches. In this scientific field, multidisciplinary cooperative work can result in quite significant discoveries.

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Appendix I

Molecular methods

Supplementary Table I – Molecular methods. Pop – population, Gor – Gorringer Bank, Dar – Darwin MV, Mek – Meknès MV. Nested PCR means that the protocol was repeated, replacing the DNA template for the PCR product. For more details, consult Molecular analyses section, page 24 (Chapter 3).

Sample no. (Pop)	DNA extration kit	DNA diluted for amplification?	PCR protocol	Temperature profile
19 (Gor)	DNeasy Blood and Tissues (Qiagen, Valencia, CA)	Yes, 1%.	A + Nested	94-4'-[95-1'-47-1'-72-1']-72-10'-15 (x35)
23 (Gor)				
31 (Gor)				
35 (Gor)	QIAamp DNA Micro (Qiagen, Valencia, CA)	No.	B	
39 (Gor)	DNeasy Blood and Tissues (Qiagen, Valencia, CA)	Yes, 1%.	A + Nested	
43 (Gor)	ISOLATE II Genomic DNA (Bioline, Taunton, MA)	Yes, 1%.	A + Nested	
44 (Gor)		No.	B + Nested	
47 (Gor)	DNeasy Blood and Tissues (Qiagen, Valencia, CA)	Yes, 1%.	A + Nested	
83 (Dar)		No.	B + Nested	
92 (Dar)		Yes, 1%.	A + Nested	
98 (Dar)	ISOLATE II Genomic DNA (Bioline, Taunton, MA)	Yes, 1%.	A + Nested	
135 (Mek)	DNeasy Blood and Tissues (Qiagen, Valencia, CA)	No.	B	
137 (Mek)	ISOLATE II Genomic DNA (Bioline, Taunton, MA)	Yes, 1%.	A + Nested	
141 (Mek)				

Appendix II

Fu and Li test for neutrality

Appendix II – Fu and Li test for neutrality

Supplementary Table II - Fu and Li tests for neutrality. Ns means not significant.¹ All individuals of *Idas modiolaeformis* including all sites in table plus LD.² All individuals of “*I.*” *simpsoni* from Setúbal, LD and Meknès.

<i>Idas modiolaeformis</i>					
Populations	D*	Stat sign (D*)	F*	Stat sign (F*)	Fs
Gor	1.083	Ns, P > 0.10	1.145	Ns, P > 0.10	3.038
Dar	-0.977	Ns, P > 0.10	-1.037	Ns, P > 0.10	2.627
Mek	-0.847	Ns, P > 0.10	-0.867	Ns, P > 0.10	4.944
NDSF	-1.379	Ns, P > 0.10	-1.505	Ns, P > 0.10	-1.653
All ¹	0.264	Ns, P > 0.10	-0.217	Ns, P > 0.10	0.275
“ <i>Idas</i> ” <i>simpsoni</i>					
Populations	D*	Stat sign (D*)	F*	Stat sign (F*)	Fs
Set	-2.879	*, P < 0.05	-3.048	*, P < 0.05	-8.72
All ²	-3.130	*, P < 0.05	-3.306	**, P < 0.02	-13.007