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Estrutura genética e conectividade entre populações do bivalve de profundidade *Acesta* em canhões submarinos do Atlântico Norte

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica da Doutora Ana Margarida Medrôa de Matos Hilário, investigadora auxiliar do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro

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palavras-chave *Acesta*, corais de água fria, mar profundo, mtCOI, rede de haplótipos

resumo

Os avanços na exploração do mar profundo mudaram a nossa visão de um ambiente calmo, sombrio e desprovido de vida, para um ambiente onde a vida apresenta adaptações para suportar condições ambientais extremas e prosperar em habitats que frequentemente albergam elevada biodiversidade, como recifes de coral de água fria. A estrutura de carbonato de cálcio segregada por estes corais fornece uma matriz tridimensional que fornece abrigo e recursos para muitas espécies se estabelecerem de forma permanente ou temporária. As crescentes pressões antropogénicas relacionadas com a exploração de recursos biológicos e minerais no oceano profundo, especialmente os impactos da pesca de arrasto demersal, resultam na destruição dessa estrutura, afetando toda a comunidade a ela associada. Os impactos nos recifes de corais de água fria e na fauna a eles associada são preocupantes o suficiente para justificar esforços globais de conservação desses habitats únicos e frágeis, principalmente através do estabelecimento de áreas marinhas protegidas (AMP's). O design de AMP's depende de estimativas de conectividade e escalas de dispersão para os taxa de interesse, medidas estas que são escassas em espécies de profundidade.

O objetivo inicial deste estudo era avaliar a conectividade genética entre populações bivalve *Acesta excavata* associadas a corais de água fria na margem europeia, tanto em recifes como em paredes verticais de canhões submarinos. No entanto, a aplicação de métodos moleculares, nomeadamente "DNA barcoding", revelou a presença de uma outra espécie deste género, associada ao coral *Lophelia pertusa* no canhão submarino de Whittard (Margem Irlandesa). Este é o primeiro relato da espécie *Acesta cryptadelphe* no Nordeste Atlântico que, até agora era conhecida apenas do Noroeste Atlântico. Este resultado inesperado reflete as dificuldades taxonómicas que ainda persistem no estudo do oceano profundo. A análise da rede de haplótipos demonstra que o fluxo genético através do Oceano Atlântico é praticamente inexistente, mas a possível existência de haplótipos não amostrados aumenta a possibilidade de existência de populações desconhecidas de *Acesta cryptadelphe* entre as duas margens do oceano, especificamente na crista meso-Atlântica.

Relativamente a *Acesta excavata*, a análise de sequências de dois ramos do canhão de Whittard mostra que, apesar de topografia e hidrografia complexas, não existem barreiras aparentes ao fluxo genético entre os diferentes ramos do canhão. A análise de haplótipos revela partilha de haplótipos entre o canhão de Whittard e a margem norueguesa e o canhão de Lisboa, sugerindo a ocorrência de um polimorfismo ancestral ou conectividade contemporânea entre os locais de estudo. Apesar de a análise de diferenciação genética não ser conclusiva, essencialmente devido ao número reduzido de sequências da Noruega e do canhão de Lisboa, os resultados obtidos permitem estabelecer diferentes hipóteses que podem ser testadas no futuro usando, idealmente, uma abordagem integrativa no estudo da conectividade entre populações. A conectividade ao longo da margem europeia pode ocorrer através da dispersão larvar, com recurso a correntes oceânicas como vias de transporte e através de populações desconhecidas que atuam como alpondras.

De forma geral, esta tese contribui com novos conhecimentos e dados relevantes para apoiar decisões de proteção de habitats vulneráveis na margem europeia.

keywords *Acesta*, cold-water corals, deep sea, mtCOI, haplotype network

abstract

Advances in the exploration of the deep sea changed our view of an environment calm, dark and barren of life, to an environment where life presents adaptations to endure the extreme environmental conditions and prosper in habitats often hosting high biodiversity such as cold-water coral reefs (CWC).

The calcium carbonate structure segregated by cold-water corals provides a 3D framework that offers shelter and resources for many species to establish, either permanently or temporarily. Increasing anthropogenic pressures related to the exploration of biological and mineral resources, especially the impacts of demersal trawling result in the disruption of this framework and ultimately affect the entire associated community. Impacts on CWC and associated fauna are serious enough to warrant global efforts to conserve these unique and fragile habitats, particularly through the establishment of marine protected areas (MPA's). MPA design depends on estimates of connectivity and scales of dispersal for the taxa of interest, which is missing for most deep-sea species.

The original objective of this study was to assess genetic connectivity between populations of the giant deep-sea clam *Acesta excavata* associated to CWC habitats in the European margin, in both reef formations and vertical walls of submarine canyons. However, the use of molecular methods, namely DNA barcoding, revealed the presence of another species of this genus associated to the cold-water coral *Lophelia pertusa* in the Whittard canyon (Celtic margin). This is the first report of *Acesta cryptadelphe* in the NE Atlantic, which until now was only known from the NW Atlantic. This unexpected result is a good example of the taxonomic issues that still persist in deep-sea ecosystems. Haplotype network analyses show that gene flow across the Atlantic Ocean is practically inexistent, but the existence of haplotypes that where not sampled raises the possibility of unknown populations of *Acesta cryptadelphe* in between the two margins, specifically in the Mid-Atlantic Ridge.

Regarding *Acesta excavata* the analyses of sequences from two branches of the Whittard canyon show that, despite the complex topography and hydrography, there are no apparent barriers to gene flow between different branches of the canyon. Haplotype analyses reveal shared haplotypes between the Whittard canyon and the Norwegian margin and the Lisbon canyon suggesting a shared ancient polymorphism or present connectivity between locations. Genetic differentiation analyses are not conclusive, especially because of the low number of sequences available for Norway and the Lisbon canyon, but allow to establish different hypotheses that can be tested in the future, ideally using an integrative approach to understand connectivity. Connectivity along the European margin may be achieved through larvae dispersal, using different ocean currents as pathways of transport, and the presence of unknown populations acting as stepping-stones. Overall this thesis contributes with new knowledge and relevant data to support decisions to protect vulnerable habitats in the deep European margin.

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1. Introduction

Ocean exploration is traced back to the early days of humankind when the sea was used as a source of resources and pathway for the discovery of new lands, but it's only in the 19th century, with the development of technology such as sounding machines, that we started exploring the ocean depths, mainly as a scientific pursuit. The first collections of deep-sea fauna, from approximately 1600 m depth were made by Sir John Ross in 1818, during an expedition to the Artic (Ramirez-Llodra et al. 2010) that is considered the foundation of deep-sea biology (Costello et al. 2010). Edward Forbes, in 1844 formulated the first theory regarding biodiversity in the deep-sea, the "Azoic Theory": after recording fewer species with increasing depth he stated that life bellow 600 m was little or inexistent (reviewed in Tyler 2003). However, contrary reports, including the collection of organisms at depths exceeding 4000 m led a group of scientists to challenge this theory and to start a series of expeditions aiming to study physical, chemical and biological processes of the deep-sea. Of these, the most illustrious is that of the HMS *Challenger*, from 1872 to 1876, that resulted in the description of over 4000 species and in the demise of the Azoic theory.

Nevertheless, by the end of this pioneering period of exploration, the deep ocean was still seen as a cold and calm place, where low biodiversity is faced with the scarcity of food, and the absence of light disables primary production. The last century was marked by increasingly sophisticated technology that allowed to characterize the physical environment of the deep sea and the collection of samples, and later the means for high-resolution observation, exploration and *in situ* experimentation that resolved several paradigms, explained biodiversity patterns and species distributions and of course raised new questions on deep-sea biology (Danovaro et al. 2014).

Temperature plays a major role on species distribution and diversity in the deep-sea. In general, temperature decreases with increasing depth, reaching a constant temperature of 2°C in abyssal plains (Thistle 2003) but can reach very elevated values in the vicinities of hydrothermal vents, where extrusive magna fluid reaching 400°C warms the surrounding water (Fornari et al. 1998), forcing organisms to present adaptations to cope with this extreme environment. Pressure increases 1 atm for every 10 m of the water column, reaching 1000 atm in the deepest areas of the ocean. Organisms need therefore to attenuate or eliminate the negative effects of pressure on their metabolism (Pradillon and Gaill 2007). The tolerance to low temperatures and to high pressures constitutes a clear barrier for shallower and warmer water organism, one that must be overcome to colonize the deep sea.

Light is also a determinant factor for the biology of the deep sea: it cannot penetrate the water column below 250 m deep, compromising the ability of primary production through photosynthesis

(Thistle 2003). Some organisms from reduced habitats (e.g. hydrothermal vents and cold seeps) resort to chemosynthesis as source of organic matter (reviewed in Van Dover 2000), but the vast majority of deep-sea organisms are dependent on external food inputs for survival. Generally, the availability of organic matter in the deep sea is low and strongly influences community composition and structure across time and scale (Gage 2003). Organic material can either be actively transported towards the deep by vertical movements of organisms like zooplankton that feed on the surface and defecate in higher profundity (Steinberg and Landry 2017) or sink passively as food falls, ranging from small particles to large carcasses, with different nutritional value (Gage 2003). The small particles or particulate organic matter (POM) are formed by erosional processes of detritus with terrestrial or shallow water origins such as plants and macroalgae and from remains of plankton and fecal pellets, representing the most important source of organic carbon in the deep sea. The action of internal tides and occasional storms disturbs the sinking of POM, increasing the horizontal food flux and originating locations of enhanced food supply (Gage 2003), ideal for the establishment of many species.

The extreme conditions of high pressure and low temperature, absence of light and low food availability seem incompatible with life (Pradillon and Gaill 2007), but the discovery of unique habitats lead to the acknowledgement of several adaptations that allow organisms to successfully colonize the deep sea. Covering about 50% of the surface of the Earth the deep seafloor (below 200 m) is now known to host a wide variety of habitats, such as cold-water coral reefs and submarine canyons that harbour high biodiversity and provides a wealth of resources (Ramirez-Llodra et al. 2010). Nonetheless, the deep sea is still one of the least studied ecosystems in the planet: it is estimated that only 5% of the deep sea has been remotely explored and a residual 0.01% has been fully studied (reviewed in Ramirez-Llodra et al. 2010). Some the biggest knowledge gaps are related with the processes that control species distribution, particularly connectivity (Danovaro et al. 2014), and there is still the certainty that a large portion of the biodiversity remains unknown (Appeltans et al. 2012).

1.1 Cold-water coral reefs and associated fauna: distribution, threats and protection

Cold-water corals (CWC) comprise 65% of the global coral diversity (Roberts et al. 2009) and occupy vast extensions of the shelf breaks, continental slopes, seamounts and canyons (Freiwald et al. 2004). However the true scale of these habitats has only become evident with recent technological advances that allow to explore deep water ecosystems (Freiwald et al. 2004; Roberts et al. 2006). CWC are represented by 4 major taxa: Scleractinia (stony corals), Antipatharia (black

corals), Octocorallia (soft corals) and Hydrozoa (hydrocorals) (Roberts et al. 2006). Sceleractinian species such as *Madrepora oculata*, *Goniocorella dumosa* or *Enallopsammia profunda*, present themselves as colonial organisms that segregate calcium carbonate to form hard skeletons in which polyps thrive (Roberts et al. 2009). *Lophelia pertusa* is the most common of reef forming cold-water coral, that distributes specially in the Northeast Atlantic (Fig.1) (Davies and Guinotte 2011; Freiwald et al. 2004; Rogers 1999; Roberts et al. 2006).

Contrary to tropical coral species, that rely in symbiotic relations with zooxanthella, CWC feeding mechanisms, according size and species, may vary between predatory behaviour or filtration of particulate organic matter from the water column (Roberts et al. 2009), taking advantage of vigorous hydrodynamic regimes through processes of up-welling and down-welling to thrive (White and Dorschel 2010). Food availability, the need of hard substrates, temperatures from 1 to 15 ºC and with oxygen concentration above 3 ml/l (Freiwald 2002; Wienberg and Titschack 2015), restrict the spatial distribution of CWC to seamounts, submarine canyons and edges of continental shelves (Purser et al. 2013) in recorded depths from 39 to 3383 m (reviewed in Roberts et al. 2009).

The processes underlying the formation of reefs are a combination of physical and biological factors, from the finding of suitable habitat to the growth rate of the coral. As the framework is being constructed, fragments break from the primary colony due to the natural weakness of attachment points, bioerosion processes resulting from the activities of organisms such as sponges and polychaetes that settle and by physical impact of large particles (Rogers 1999). The living coral occupies the more superficial part of the reef and continues to grow laterally, with the dead coral frame serving as new attachment sites, spreading from a few meters to several km (Freiwald et al. 2004; Wheeler et al. 2007). For example, in the Sulla Reef (Mid Norway) the *Lophelia pertusa* framework reaches 30 m height, 400 m width and 13 km length (Hovland et al. 2005) that considering the *L. pertusa* growth rate (4-25 mm/year) translates in to a reef that is 8000 to 10000 years old (Rogers 1999).

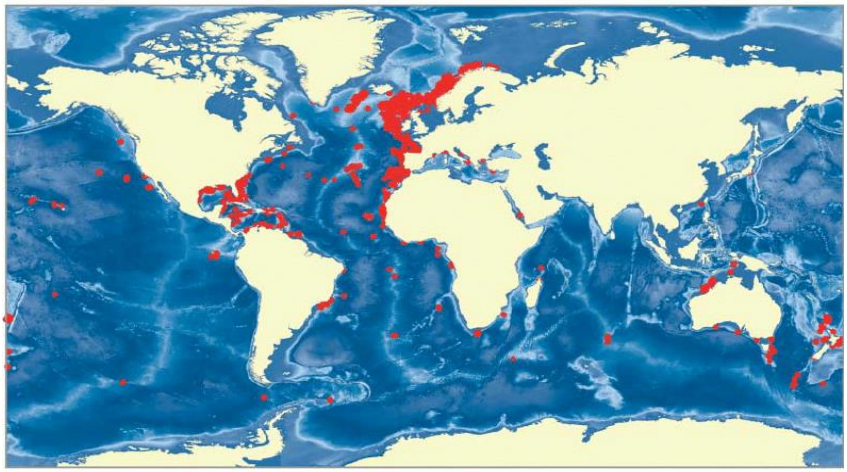

Figure 1 - Global distribution of cold-water coral reefs (Roberts et al. 2006)

CWC reefs are considered hotspots of biodiversity (Roberts et al. 2009) as they provide habitat and resources for a large number of different organisms (Wienberg and Titschack 2015), allowing temporary or permanent establishment (Roberts et al. 2006). There are more than 1300 reported species living in association with *Lophelia pertusa* reefs: the biofilm covering the hard framework is food source for small crabs, gastropods, echinoids and sea stars; the high abundance of these small species turns these habitats in hunting grounds for benthic predators such as molluscs and crustaceans, ultimately becoming preys as well; fish, including species with commercial value, such as roudnose grenadier, orange roughy, leafscale gulper shark or Portuguese dogfish (Hall-Spencer et al. 2002), may associate permanently to the reef, for foraging and/or in search of refuge and nursery ground (Costello et al. 2005). Sponges, polychaetes, anemones, bivalves and hydroids colonize spaces between coral branches where filter feeders take advantage of food particles retained in the framework (Henry and Roberts 2017).

Since the 1980, the deep sea has become increasingly targeted for exploration of its biological and mineral resources, but only with the development of sophisticated camera equipment, the true extent of the damages has been revealed (Roberts et al. 2009; Freiwald et al. 2004). CWCs are particularly affected by anthropogenic activities because of their slow growth, fragility (Hall-Spencer et al. 2002) and extended recovery periods (Heifetz et al. 2009). Therefore, according to United Nations General Assembly (UNGA 2008) and the Food and Agriculture Organization (FAO 2009) they are considered vulnerable marine ecosystems (VME) that are characterized by their: i) uniqueness in the ecosystem or rarity of species; ii) functional significance of the habitat; iii) fragility; iv) long recovery period; v) structural complexity (Murillo et al. 2011).

Fishing activities such as bottom trawling (Fig.2), dredges and weights to sink nets are major threats to coral reefs and associated biological communities (Rogers 1999) as they smash, flatten and disrupt the carbonated structures, affecting the 3D complexity that contributes for the elevated biodiversity and presence of endemic species (Freiwald et al. 2004; Roberts et al. 2006). Other concerning impact are the resuspension of sediments that leads to stress and to burying of benthic organisms, which results in the alteration of the substrata, compromising the availability of suitable settlement habitats (Roberts et al. 2009; Rogers 1999).

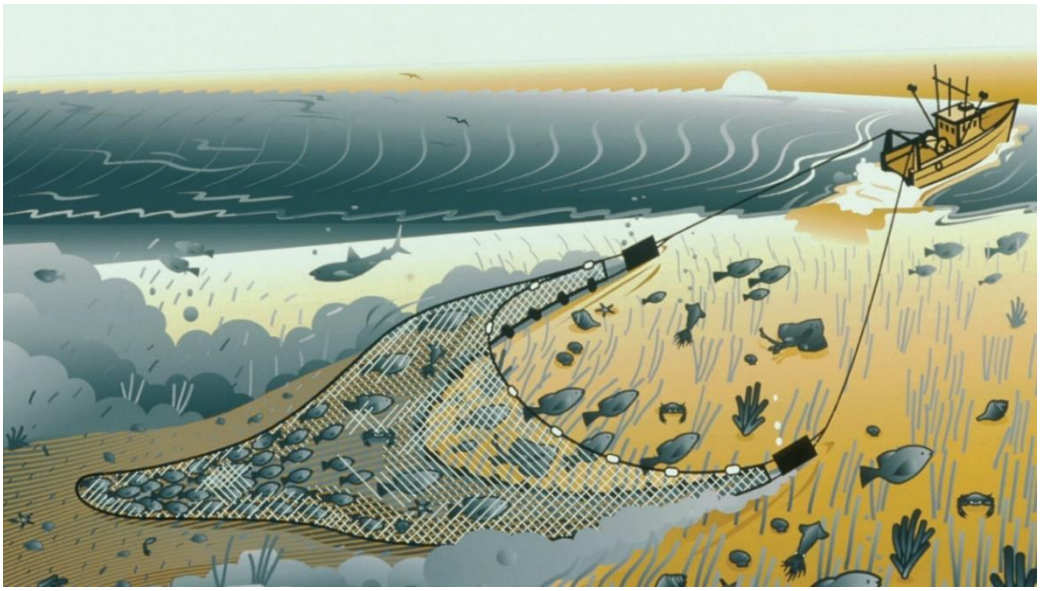

Figure 2 - Representation of the impacts of bottom-trawling on the seabed (Freiwald et al. 2014)

As mineral resources on land and shallow waters are depleting, oil companies began to explore the seabed at increasing depths, producing sediment plumes and drilling muds that may result in the smothering of CWC species and associated benthic organisms and that may release toxic compounds that compromise the survivorship of both benthic and pelagic biodiversity (Freiwald et al. 2004). Further, Freiwald et al. (2004) refers climate change and rapidly increasing levels of CO_2 as threat to the future of corals communities, as the calcification rate may be slowed down consequently compromising the framework formation and habitat construction (Freiwald et al. 2004).

From the current threats to CWC arises the necessity of protection, especially regarding the negative impacts of demersal bottom trawling, the major responsible for CWC destruction (Clark et al. 2016). In order to protect the existing reefs, Norway has, since 1999, forbidden fishing activities around the Sula Reef, within its ZEE, with other countries such as the UK, Ireland and Portugal (in the Azores) implementing similar measures. Currently, the protection of VME's follows the guidelines and regulations established by UNGA (2006 and 2008) (Weaver et al. 2011) and FAO (2009) for sustainable management of deep-sea fisheries. Protecting CWC areas does not only protect the corals species but all the community established in the habitat provide by the reefs. The identification of VME's and protective measures culminate in the implementation of Marine Protected Areas (MPA's), in an effort to preserve the existing biodiversity and habitats in its most pristine state (reviewed in Goodsell and Underwood 2009). The legislation regarding the restriction, regulation or prohibition of habitat destruction/modification subdivide MPA's into 3 types: i) total closure to anthropogenic activities, ii) limited catches and iii) temporary impediment to human

actions. Corridors between MPA's are also essential for populations as several species present both benthic and pelagic life stages, with larvae of some species (e.g. scleratinian corals and bivalves) using oceanic currents to disperse. Allowing the exchange of individuals/genes prevents inbreeding and is essential in the preservation and persistence of the different populations (reviewed in Cowen and Sponaugle 2009; Sponaugle et al. 2002).

Knowledge regarding coral biology and ecology is fundamental towards the protection of these VME's that, when combined with studies of other species of the reef community and knowledge on hydrodynamics, allows a better design of networks of MPA's, including their location, size and management measures (Freiwald et al. 2004).

1.2 The deep-sea file clam *Acesta*

Species of the genus *Acesta* (Bivalvia: Limidae) are some of the most common species associated with cold-water corals (Fig.3). Agglomerations of these giant clams introduce additional spatial complexity to the 3D framework created by the coral, providing further shelter from currents, predators and suitable conditions to particular life-stages (Brooke et al. 2017; Gagnon et al. 2015; López Correa et al. 2005).

Figure 3 - Association between *Lophelia pertusa* and *Acesta excavata* (photo: STATOIL ASA, Norway)

The first fossil records of *Acesta* are dated from the late Mesozoic and Cenozoic eras, encompassing the margins of the supercontinent Gondwana and latter spreading from India, southern Chile, Japan and Hungary, with more than 30 documented species (López Correa et al. 2005). Nowadays, *Acesta* species dominate bathyal habitats, attached to rocky substrata, cliffs and

overhangs and are segregated by water masses characteristics (e.g. temperature, oxygen content and salinity), which is reflected in the species distribution at different depths. In the Eastern Pacific margin, Clague et al. (2012), reported *A. sphoni* occurring in shallower depths from 545 to 860 m, in warmer and less oxygenated waters, and *A. mori* in colder waters, with greater oxygen concentration in depths between 1000 and 2000 m, with a maximum of 2450 m in the vicinity of a hydrothermal vent (Walz et al. 2014) In the Northwest Atlantic, more specifically in the Gulf of Mexico, *A. bullisi* can be found from 400 to 800 m depth along with *A. oophaga*, the only species of the genus *Acesta* that adopts a parasitic/predator behavior by living bissally attached to the tubeworm *Lamellibrachia luymesi* and feeding on its eggs (Burris et al. 2014; Clague et al. 2012; Järnegren et al. 2007). *Acesta cryptadelphe*, which designation derives from "crypto" as hidden and "adelphe" as sister due to morphological similarity to a Northeast Atlantic species, is the most recently described species of the genus and is found in several sites of Northwest Atlantic (Gagnon et al. 2015). Occurring in overhangs, rocky walls and in isolated outcrops at depths between 600 and 1241 m, *A. cryptadelphe* was found co-occurring with *Desmophyllom dianthus, Primnoa resedaeformis* and other cold-water corals but not with *Lophelia pertusa* as typically occurs with other species of *Acesta*.

Acesta excavata (Fabricious 1789) is the Northeast Atlantic representative of this genus. First documented in the Norwegian fjords its current distribution extends from Greenland to the Mediterranean Sea, Ireland shore and Azores, often associated with corals *Lophelia pertusa* and *Madrepora ocullata* (Clague et al. 2011; Järnegren and Altin 2006; López Correa et al. 2005). Temperature extremes for this species vary between 3 and 13 ºC, allowing it to survive in deeper water (~3200 m) or in areas as shallow as 40 m. However *A. excavata*, which displays the highest filtration rate of the genus (Järnegren and Altin 2006) shows a clear tendency to occupy intermediate water layer boundaries, where enforced circulation and nutrient enriched water column provide the most favorable habitat for this species to thrive (Clague et al. 2011; Järnegren and Altin 2006; López Correa et al. 2005).

As the other species, *Acesta excavata* possesses a slightly oval thin white shell, full of radial ribs and a pale orange soft tissue with tentacles that emerge from the folded mantle margin through the existing gap. The foot allows the animal to crawl and with the assistance of strong byssus threads to attach to the substrata (reviewed in López Correa et al. 2005). Data gathered by Järnegren et al. (2007) suggests cases of protandric hermaphroditism as females smaller than 9 cm were not recorded and their percentage in the total population increased with individual size that can reach 20 cm in mature adults. Fertilization occurs externally resulting in buoyant embryos and lecitothrophic larvae that cannot actively swim and disperse by action of oceanic currents (Järnegren et al 2007). After settlement, larvae suffer metamorphosis to the adult stage. The growing shell acquires radial ribs at a steady rhythm, which allowed to calculate a life spawn from 50 to 80 years for this species (López

Correa et al. 2005). Because of its long life *A. excavata* is considered an interesting case study for reconstruction of past seawater environmental parameters, providing similar results to those obtained from analyses of coral structures, but requiring easier analytical methods and more straightforward interpretations (López Correa et al. 2005).

Currently, the compilation of studies on the genus *Acesta* provide information relative to past and recent distribution (e.g. López Correa et al. 2005 and Walz et al. 2014) and biological traits such as reproductive cycles, larval mobility (Järnegren et al. 2007), filtration rates (Järnegren and Altin 2006) and feeding mechanisms (Clague et al. 2012) for most of its species. A major knowledge gap on the biology of this genus is related to the lack of studies of population connectivity. This knowledge is of utmost importance to infer to which degree patchily distributed populations depend on each other to persist or to recover from disturbances, and is crucial towards the preservation of these giant clams.

1.3 The Whittard canyon

Submarine canyons have been previously highlighted as preferable locations for the establishment of cold-water coral and associated communities. These topographic structures are formed by continuous erosion processes of the continental shelfs and slopes that are derived from glaciation episodes and present day submarine landslides (reviewed in Amaro et al. 2016; Harris and Whiteway 2011). Canyons are characterized by intricate patterns of hydrography, with water column stratification and currents above and within the structure, sediment and organic matter transport and accumulation often disturbed by internal tides (Bosley et al. 2004). The stepped topography of hard substrate walls with cliffs, overhangs and gullies that confers protection from the strong tides, contrasts with the muddy bottom sediment, conferring habitat heterogeneity for the establishment of several niches of biological communities that also advantage of enhanced primary production and up-welling through the interaction of structural and hydrodynamic characteristics (Fernandez-Arcaya et al. 2017; Genin 2004). The settlement of corals and reef formation result in elevated micro- to megafauna biodiversity and provide essential habitats for several life-stages of benthic and demersal species, including fish and shellfish of commercial interest (Amaro et al. 2016; Vetter and Dayton 1999).

The Bay of Biscay (Fig.4a) is a sedimentary basin between Spain, France and England, covering a total area of 900000 Km^2 with a Northwest orientation (Cunningham et al. 2005; Mulder et al. 2012). Extending for 1000 km and water depths reaching 4975 m, the slope is dominated by canyons that connect the continental shelve to the deep fans acting as conduits of biogenic and

lithogenic sediment transportation (Fernandez-Arcaya et al. 2017; Mulder et al. 2012; Puig et al. 2014). The Celtic margin limits the Bay of Biscay in the north, extending for 250 km from Goban spur to the Berthois Spur with the Irish Sea and English Channel as limitations (Bourillet et al. 2006).

The Whittard canyon (Fig.4b) is the most western of approximately 35 canyons of the Celtic margin (Mulder et al. 2012), located 300 km apart from the shore line of the British Isles (Reid and Hamilton 1990); it represents an intricate sediment transport pathway that comprises the canyon branches and the Whittard channel, at the mouth of the canyon, that feed sediment mud into the Celtic fan at 4500 m deep (Amaro et al. 2016; Cunningham et al. 2005; Reid and Hamilton 1990). The canyon system is composed by four main V-shaped branches with a NNE-SSW orientation (Cunningham et al. 2005) that connect the relatively flat continental shelf at 200 m, to the deep channels, with intricate gullies and scars of collapsed walls at the head of the canyon and slope angles up to 40 degrees as depth increases (Stewart et al. 2014).

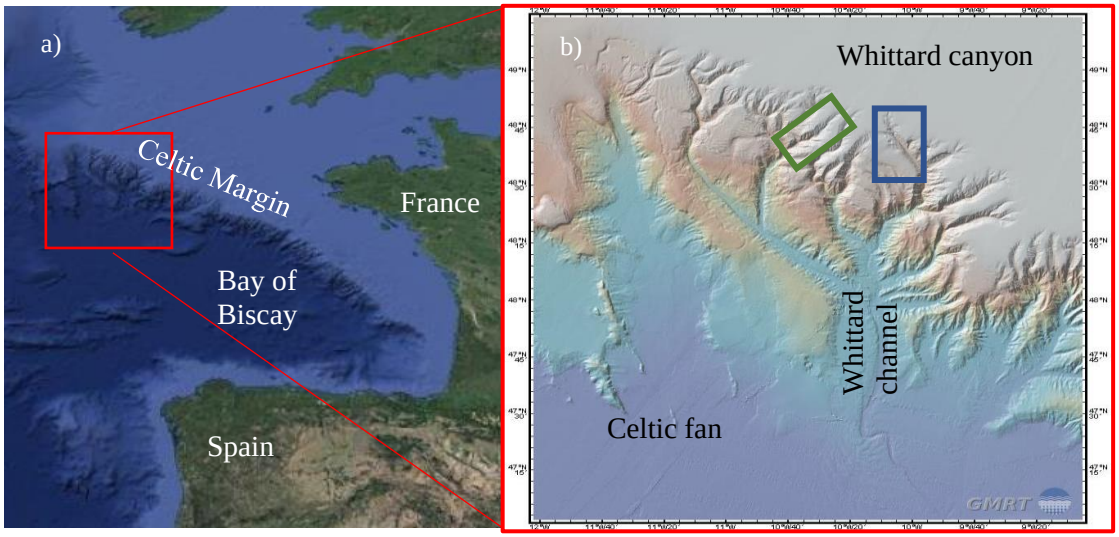

Figure 4 – a) Localization of the Whittard canyon in the Celtic margin and b) a close picture of the branches, the Whittard channel and the Celtic fan. The green square signalizes the Acesta branch and the blue square the location of Lophelia branch.

The upper parts of the canyon are composed by coarse granules of lithogenic material, suggesting a transportation shelf-fan oriented; as depth increases, a decrease in lithogenic concentration and alteration to muddy seabed is observed (Cunningham et al. 2005; Stewart et al. 2014). Hydrodynamics inside the canyon is strongly influenced by the location of the Celtic margin, between the two main Atlantic gyres, generating high-energy tides due to interaction between water masses. The shallower water mass, the Eastern North Atlantic Water (ENAW) spreads to a depth of 800 m, with temperature between 4 and 6°C and a poleward circulation orientation. Bellow, the Mediterranean Outflow Water (MOW) extends to 1200 m, with temperature about 10°C, hotter than

the boundary water masses. The deepest layer is originated by Labrador Sea Water masses (reviewed in Amaro et al. 2016; Dullo et al. 2008).

The interactions of water masses and consequent mixing, internal tides and waves, that deflect on the canyon walls are responsible for resuspending the bottom layers of sediments, resulting in mixing and turbulence of organic matter (nepheloid layers) that ultimately influence the biological and ecological traits of benthic organisms. Mixing of water layers also poses a major influence to nutrient fluxes, resulting in enhanced primary production (reviewed in Amaro et al. 2016).

Biological communities are diverse across the four main branches of the Whittard canyon and vary according to the depth gradient, oxygen content and food availability. Regarding megafaunal communities, Ismail (2016) reports similar species richness on the western and eastern branches of the Whittard canyon, even though abundance decreases in an east-west direction. Robert et al. (2014), concluded that the higher abundance was restricted to depths shallower than 1000 m with small peaks at 2200 and 3000 m, while the peak of richness occurred at 1200 m.

Cold-water corals can be found between 880 and 3300 m depth, with occurrences of *Lophelia pertusa*, *Primroa sp*. and *Acanella sp.*, among others. The highest density of *L. pertusa* (70% of biological coverage) was reported by Huvenne et al. (2011) in an overhanging vertical wall, in the so called Lophelia branch (Fig.4b), from 1350 to 2448 m deep. Many other species such as anemones, sea stars and the bivalve *Acesta excavata* were found in association with *L. pertusa*. Several reef structures made by *L. pertusa* and *Madrepora oculata* were also documented in the eastern middle branch between 400 m and 1500 m (Amaro et al. 2016; Robert et al. 2014). In another branch, the Acesta branch (Fig.4b), *A. excavata* also appears in depths ranging from 633 to 762 m, forming another vertical wall assemblage with the bivalve *Neopycnodont zimbrowii* (Johnson et al. 2013). The dominance of filter feeders on vertical substrata is suggested by several authors as a mean to avoid the mixing and turbulence of sediment layers below, as well as a better suited position regarding food availability refugee and nursery (Huvenne et al. 2011; Johnson et al. 2013). At greater depths of the canyon and in the Whittard Channel, dense aggregations of holothurians have been observed (Amaro et al. 2015).

Anthropogenic threats and impacts in the Whittard canyon are similar to others recorded in several other canyons and seamounts. Although the canyon is located far from shore, litter contamination was still recorded and, of its total, 28% was lost fishing gears (Pham et al. 2014). Bottom trawling poses the major threat towards the benthic communities of Whittard canyon as resuspension of the fine and loose sediment in the upper parts can result in enhanced nepheloid layers that can easily burry sessile organisms and smoother filter feeders with non-nutritional particles (Puig et al. 2012; Wilson et al. 2015). The 2018 OSPAR report highlights that although the protection of Bay of Biscay is under jurisdiction of the bordering countries (Spain and France) there are no

protected areas currently implemented. The thrive of populations is assured through several processes that connect different populations. For the preservation of the cold-water corals and associated fauna in this area it is imperative to understand how populations from different branches may connect to each other and the degree of dependence to other populations from surrounding areas. To design and implement a network of MPA's including the Whittard canyon it is essential to study and target the populations that contribute to the exchange of individuals, ensuring the necessary gene flow that allow these populations to be maintained and survive disturbance.

1.4 Population connectivity

Population connectivity can be simply resumed in the exchange of individuals of a certain species between local sites or subpopulations, creating a migration net defined as metapopulation (Cowen and Sponaugle 2009). For the persistence and resilience of the populations, the number of births and immigrants must be superior to the number of deaths and emigrants. In cases of inexistent migration, the population is described as closed, resulting in reproduction of closely-related individuals and in-breeding, compromising its persistence (Allendorf and Luikart 2007; Cowen and Sponaugle 2009). Populations where random exchange of individuals occurs are characterized as open and in a state of panmixia (David and Loveday 2017).

Exchange of individuals cannot be dissociated from the exchange of genetic material. Geographically distant sub-populations can contribute with migrants, although is to be expected to have genetic variation within and between them so, the metapopulation structure should reflect patterns of such variation (Allendorf and Luikart 2007).

Evolutionary forces are the main responsible for genetic variation across geographic areas: migration, genetic drift and natural selection. In the absence of such forces in a large population, a state of equilibrium would be installed, the Hardy-Weinberg equilibrium, decreasing the rate of mutations that results in the transmission of the same genetic information to future generations (Chen 2010). Any variation recorded in a population is therefore a deviation of this principle (Allendorf and Luikart 2007).

Population structure studies must consider the forces of genetical differentiation according to the population isolation: when closed, the only forces in action are genetic drift and random matting. Considering open populations, individual migration also creates a gene flow between them which tends to genetically homogenize individuals even with long distance separation. On the other hand, genetic drift tends to change allele frequency on the populations and natural selection will act in favour of the fittest, causing divergence in the metapopulation structure (Allendorf and Luikart

2007). When considering that the only evolutionary forces causing genetic variation are gene flow (migration) and genetic drift, not natural selection, the population follows a neutral evolution model (Hartl and Clark 1997).

Population structure and connectivity can be described by four models that relate gene flow and geographic distance between populations. The first model is the "island model" which the simpler version contemplates equal sized populations and genetic exchange that contribute to a gene pool (Fig.5a); the "main-island" version (Fig.5b) considers a panmictic population surrounded by smaller ones, that receive genes from the first mentioned. The "stepping-stone" model (Fig.5c) considers an intermediate case of the two previously mentioned models, when the gene flow occurs only between surrounding populations, with same size and migration rates. The "isolation by distance" model (Fig.5d) is influenced by the size of the populations and how far apart they are located from the closest one (Hey and Machado 2003).

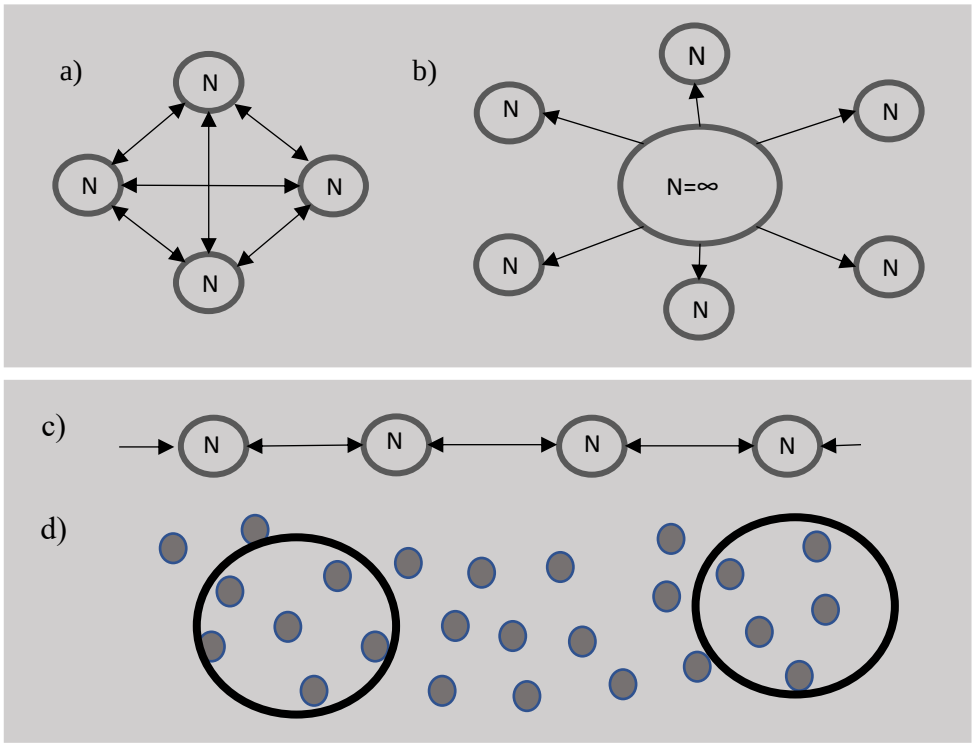

Figure 5 - Models of population structure. Schemes a) and b) represent the two versions of the "island model": a) the classical b) the "main-island"; c) "stepping-stone model"; d) isolation-by-distance" model. Image adapted from (Allendorf et al. 2007).

The study of population connectivity is most effective when both genetic connectivity and demographic connectivity are considered. The demographic data informs about how survival rates and population growth are influenced by migration movements. Genetic connectivity depend on the number of emigrants that effectively enter a population and how evolutionary processes occurs

within (Lowe and Allendorf 2010). Regarding marine population connectivity, studies must also consider species with life cycles that take place in two different ecosystems. For 80% of marine species, the life cycle comprises a larval stage and an adult stage. The larvae in the water column are dispersed by action of ocean currents until the finding of suitable habitat in which they settle. The settlement process is followed by a metamorphosis from larvae to a benthic adult that start to mature (Bhaud and Duchêne 1995) and acquires reproductive capacity (Jenkins et al. 2009). Mature organisms that recruit in the population are the responsible for reproduction, untimely influencing the production of larvae (reviewed in Cowen and Sponaugle 2009), the responsible for migrations between populations (Pineda et al. 2007).

Fecundity, the number of offspring that a female is capable of producing in a determined period varies according to species (Ramirez-Llodra 2002) and is the first major influence in larval production; it is regulated by intrinsic and extrinsic factors such as food availability, size, age, competition and environmental stressors, as well as gametes quality. The other main influence in larval supply is fertilization success, which may not reflect the fecundity of adult specimens. In broadcast spawners, the main constrain to successful fertilization is the dilution of the gametes in the water column (Metaxas et al. 2002), conditioning their encounter due to local hydrodynamics. After fertilization, the dispersal process continues under influence of physical and biologic factors. The main physical influencers are currents and water stratification (due to alteration of parameters as temperature and salinity) which limits the distribution of larvae. Biological factors include vertical migrations, swimming ability and predation (Cowen and Sponaugle 2009).

Finding suitable habitat depends on the time that larvae can remain dispersing in the currents, the pelagic larval duration (PLD) (Shanks et al. 2003; Pineda et al. 2007). PLD is determined by the species and the environmental features that larvae encounter, can last one to several days and allow the larvae to disperse hundreds of kilometres (Cowen and Sponaugle 2009; Shanks 2009). Current velocity data, PLD and biophysical variables are fundamental for models of dispersal distances (Cowen and Sponaugle 2009). Dispersal processes ends when the larvae find a suitable habitat and settles as competent part of the populations (reviewed in Cowen and Sponaugle 2009). Mineral composition of the substrata, hydrodynamic features and topography play a determinant role (reviewed in Jenkins et al. 2009) as the responses to biological and physical hints that allows the acceptance or rejection of the site (reviewed in Jenkins et al. 2009). In some species, the larval settlement site does not correspond to the final adult habitat as juveniles may search protection, distinct types of food and reduced competition (reviewed in O'Connor 1993). If habitat is not ideal, larvae will detach from the substrata and back to the water column until settlement, followed by the recruitment phase where organisms contribute to population persistence and resilience.

1.5 Framework and objectives

The study of connectivity in the deep sea faces many challenges inherent to the vast and complex fluid environment, enhanced by barriers of accessibility and sampling constrains (Baco et al. 2016; Hilário et al. 2015). Even though large topographic structures such as abyssal plains and slopes in the continental margin are contiguous, the geological, physical, and geochemical properties of the seafloor and water column form unique featured habitats that support specific faunal communities (Ramirez-Llodra et al. 2010). With such variation of characteristics, many deep-sea populations are spatially fragmented and tend to become even more scattered, consequence of the posing threat of anthropogenic resource exploitation and extraction. Ultimately, the persistence and recovery of these populations is dependent on processes of connectivity (reviewed in Cowen et al. 2007).

Most marine benthic species have complex life cycles that include a pelagic larval state, and sessile/sedentary adulthood and therefore connectivity between populations is dependent on larval transport. Consequently, there has been an increased effort in the identification of larval paths and the spaciotemporal variation in intensity, direction and dispersal distance (Cowen et al. 2007), requiring an understanding of the biological and physical factors that regulate the small sized larvae dispersion, settlement and recruitment (Hilário et al. 2015). Due to relative inaccessibility and heterogenicity of the deep-sea environment, most connectivity studies focused on habitats with high abundance of animals such as hydrothermal vents and seamounts (Vrijenhoek 2010; Young and Shank 2010).

The current threats to CWC and their status as vulnerable marine ecosystems (Murillo et al. 2011) highlight the necessity of studies of population connectivity and genetic structure as basis for conservation measures and predicting recovery scenarios. Additionally, population connectivity studies regarding species that occur in the habitats provided by CWC may contribute as a warranty for global preservation efforts.

Marine protected areas, corridors and associated legislation play an increasingly important role in the protection of vulnerable habitats, in an effort of creating a balance between the ecological needs of the biological communities and the economic, social, political status of implementation countries (Cowen and Sponaugle 2009). Such conservations and management measures are now being expanded to international waters, generally deeper and where national laws do not apply (Wedding et al. 2013). In the Northeast Atlantic, the OSPAR report on Network of Marine Protected Areas (2018) reveals a coverage area of 864,337 km^2,counting with 486 MPA's implemented within the ZEE of Contracting Parties and 10 off limits of National Waters (OSPAR 2018). Because the persistence of populations will depend on either sufficient large local replenishment in a single patch

or sufficiently strong connectivity among patches (Burgess et al. 2014), studies of connectivity of key species, including oceanographic, hydrographic and ecological data, are essential for the design of MPA's.

The Whittard canyon and surrounding area, in the Celtic Margin, is reported as a suitable site for cold-water coral establishment, with reports of *Lophelia pertusa*, and where the association between corals and the giant file clam *Acesta excavata* is abundant (Johnson et al. 2013; Huvenne et al. 2011). Because of their long lifespan, slow growth and late reproductive maturity species of the genus *Acesta* are particularly vulnerable to habitat damage by anthropogenic activities. The capacity of the populations to recover after disturbance is dependent on the processes of population connectivity that, in similarity to CWC, is a field that is yet unexplored (López Correa et al. 2005; Roberts et al. 2006). The opportunity to sample, with a Remotely Operated Vehicle (ROV), two branches of the Whittard canyon where the association between CWC and *A. excavata* had been reported (Huvenne et al. 2011) allows for the first time to study the genetic structure of this species within the Whittard canyon, as well as along the European margin. The objectives of this dissertation are to: 1) investigate genetic diversity of the specimens collected in the Whittard canyon and 2) assess genetic connectivity between population from different sites in the European margin. The results of this study will increase the knowledge on the spatial scales of connectivity between CWC habitats and contribute to understand population persistence in these systems. Ultimately, such knowledge also builds the basis for the design of reserve networks in the deep sea and informed conservation policy decisions.

2. Material and methods

This study includes data from specimens collected in the Whittard canyon (Celtic Margin) and the Lisbon Canyon (Portuguese margin), NE Atlantic, as well as sequences of *Acesta* spp. available in GenBank (www.ncbi.nlm.nih.gov).

2.1 Sampling sites and collection

Specimens of *Acesta* were collected from two branches of the Whittard canyon (Fig.4a) that were chosen based on population densities observed in previous expeditions. The first sampling site, hereby called "Acesta branch" (Fig.4b) is characterized by walls dominated by *Acesta*, as reported in Johnson et al. 2013, and the second site, the "Lophelia branch" (Fig.4b) is composed of walls covered by cold water corals, mainly *Lophelia pertusa*, with low abundance of *Acesta* specimens as described in Huvenne et al. 2011. Samples were collected with the ROV *ISIS* (Fig.6) during the JC125 Cruise (09/08 – 12/09/2016, Huvenne et al. 2016) on board the research vessel RV *James Cook*. Collection was performed in multiple dives at different depths and in the case of the Acesta branch, from two walls, East and West (Table 1).

Figure 6 - Collection of *Acesta* spp. in the Whittard canyon using the ROV ISIS, during the JC125 cruise.

Specimens from the Lisbon canyon were collected using the ROV *Luso* on board the research vessel NRP *Almirante Gago Coutinho* during a test dive in June 2015 at approximately 1400 m depth (Table 1). The Lisbon-Setubal canyon system is one of the largest incisions of the continental shelf of west Iberian margin and supports high biodiversity including cold water corals and associated communities (Cunha et al. 2011; Weaver et al. 2009) (Fig.7).

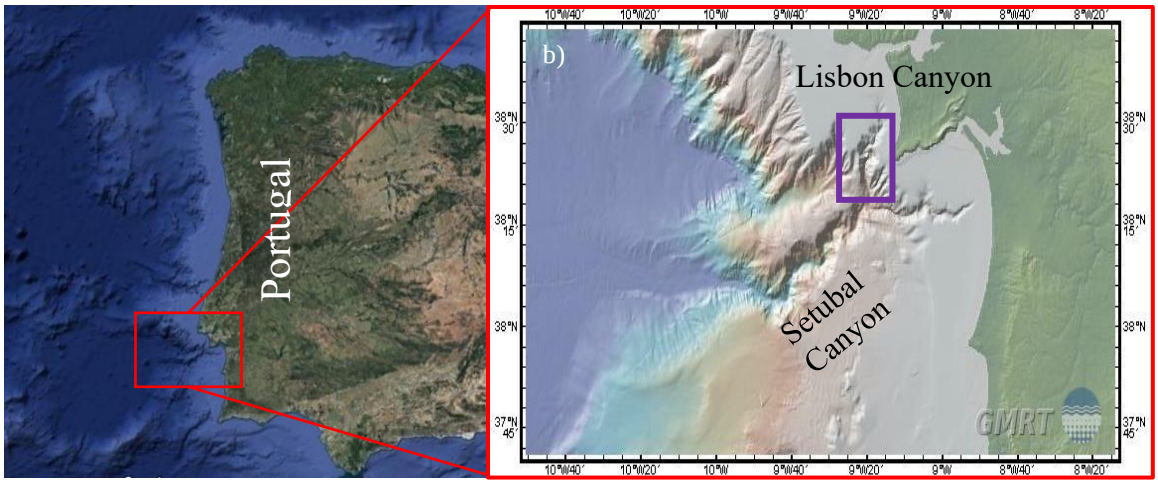

Figure 7 – a) Localization of Setubal-Lisbon canyon system in Portuguese shoreline and b) a close picture of the canyon system with highlight of the Lisbon canyon.

Table 1 - Sampling details. N: number of individuals used for DNA extraction.

Canyon	Branch	Wall	Sampling site	Coordinates (Lat / Long)	Depth (m)	N
Whittard	Lophelia	West	LWest480	48° 44.225' N / 10° 05.416' W	480	11
			LWest1310	48° 39.126'N / 10° 02.126' W	1310	17
	Acesta	West	AWest540	48° 45.881' N / 10° 27.683' W	540	8
			AWest700	48° 45.027' N / 10° 28.564’ W	700	12
		East	AEast700	48° 45.564’ N / 10° 27.514' W	700	23
Lisbon			Lis	38° 22.716' N / 09° 21.039' W	1420	2

All collected individuals were measured on board (data not relevant for this study) and a piece of the adductor muscle of each individual was dissected and preserved in ethanol 95% for molecular analyses.

2.2 Cytochrome-c-oxidase sequencing

Genomic DNA was isolated using ISOLATE II Genomic DNA kit (Bioline, UK) following the manufacturer's protocol. Segments of approximately 500 to 750 base-pairs (bp) of mitochondrial cytochrome-c-oxidase subunit I gene (COI) were amplified with primers based on regions conserved in invertebrates (Folmer et al. 1994), specifically LCO1490 and HCO2198. PCR was conducted in 20 µl reactions that included 1µl of DNA, 15.4 µl of MiliQ water, 3.1 µl of 5x MyTaq ^{DM} (Bioline, UK) reaction buffer, 0.2 µl of each primer (100NM), 0.1 µl of 5u/ µl MyTaq ^{DM} DNA Polymerase (Bioline, UK) and sterile MiliQ water to achieve the final volume. Amplifications were performed on a Biometra TProfessional TRIO thermocycler submitting the samples to 94 °C for 4 minutes followed by 35 cycles of 95 °C for 1 min, 47 °C for 1 min and 72 °C for 1 min, with a final extension of 72 °C for 10 min and awaiting temperature 15 °C. PCR products were visualized through an electrophoreses gel prepared with 0.8g of agarose, 80 ml of TAE x1 buffer and 4 µl of SyberGreen (NZYTech). Hyperladder^{TM} 50 pb (Bioline, UK) was used as molecular ladder and PCR images were obtained using BIO-RAD Molecular Imager® ChemiDoc^{TM} XRS+. A small set of samples required a dilution of x10/x100 and new amplification, in order to obtain a clear observation of PCR products. PCR products were purified with ISOLATE II PCR and Gel Kit (Bioline, UK) and sent to Eurofins Genomics lab (Germany) for bidirectional sequencing. DNA sequences were trimmed, paired and clipped using Biolign Sequence Alignment Editor version 4.0.6.2 (Hall 1999). All sequences were deposited in GenBank and the accession numbers were utilized as sample identification in the phylogenetic tree.

2.3 Phylogenetic analyses

A total of 142 sequences, 73 obtained in this study (71 from Whittard canyon and 2 from Lisbon Canyon), to which Blast at NCBI was performed and 65 published in Genbank were analysed to confirm the identity of the collected specimens. A total of six species of *Acesta* were included in the analyses and published sequences of the three species of the Limidae family were used to represent the outgroup taxa. The sample location and accession number of the sequences retrieved from Genbank are listed in Appendix I.

The correct translation from nucleotide to protein sequences was assured in EMBOSS online tool Transeq (Li et al. 2015). A ClustalW Multiple Alignment with 1000 bootstraps was performed in Biolign to align all sequences. A Best Fit Model was run in the software MEGA X (Kumar et al. 2018). Phylogenetic maximum likelihood tree (ML) was also performed in this software, selecting 1000 bootstraps, Tamura and Nei (1993) as nucleotide substitution model (result of best fit model),

4 discrete gamma categories for rate variation among polymorphic sites, all codon positions and Nearest-Neighbour–Interchange (NNI) for inference options of ML heuristic models. Pairwise genetic distances between groups was determined.

2.4 Haplotype diversity and genetic structure

The software DnaSP 5.1 (Librado and Rozas 2009) was used to calculate the number of polymorphic/segregating sites (S); total number of mutations (Eta); number of haplotypes (h); haplotype diversity (Hd) with respective variation, standard deviation and nucleotide diversity (π) of each sampling site. Tajima D and Fu and Li tests were also performed in this software in order to validate neutral evolution. To estimate the relationship among haplotypes, a network was generated with the software Network 5.0.1.1 (Fluxus Technology Ltd, since 1999) using a median-joining method (Bandelt et al. 1999) and having in consideration information obtained from Best Fit Model run in MEGA X. Genetic structure was accessed in Arlequin v. 3.5.1.2 through AMOVA between the sampling sites within the Whittard canyon (k=1) and between the Whittard canyon, the Lisbon canyon and the collections sites of the sequences available in GenBank. Significance tests were performed with 1023 permutations. Fixation index were calculated in order to infer genetic distances between sampling sites and thus, population connectivity.

3. Results

3.1 Molecular identification

The mitochondrial COI gene was successfully extracted, amplified and sequenced from all the collected specimens (Table 2). The phylogenetic analyses placed the collected specimens into two different clades (Fig.8), one composed of *Acesta excavata* with four specimens from Norway (Järnegren et al. 2007), all the individuals collected in the Acesta branch, the individuals collected in the shallower depth of the Lophelia branch, and the two samples from the Lisbon canyon. The second group includes the individuals collected at 1310 m on the Lophelia branch and five individuals of *A. cryptadelphe* collected in the Northwest Atlantic (Gagnon et al. 2015). Pairwise differences between species are shown in table 3. These results indicate the existence of two different species occurring in the Whittard canyon: *A. excavata* in both branches above 700 m and *A. cryptadelphe* below 1300 m depth on the Lophelia branch.

Table 2 - Species identification and GenBank accession numbers of the specimens.

Species	**Sampling site**	**N**	**Accession number**
Acesta excavata	LWest480	11	MN272033-MN272043
Acesta cryptadelphe	LWest1310	17	MN271973.MN271989
Acesta excavata	AWest540	8	MN271990-MN271997
Acesta excavata	AWest700	12	MN272021-MN272032
Acesta excavata	AEast700	23	MN271998-MN272020
Acesta excavata	Lis	2	MN272044; MN272045

Table 3 - Nucleotide sequence divergence (p-distance) between Limidae sequences used in this study.

	1	2	3	4	5	6	7	8	9
1 *Acesta cryptadelphe*									
2 *Acesta excavata*	0,1474								
3 *Acesta bullisi*	0,1194	0,1162							
4 *Acesta oophaga*	0,1246	0,1100	0,0585						
5 *Acesta sphoni*	0,1723	0,1368	0,1204	0,1186					
6 *Acesta mori*	0,1073	0,1005	0,0612	0,0519	0,1192				
7 *Lima lima*	0,5505	0,5076	0,5227	0,5625	0,5229	0,5294			
8 *Lima loscombi*	0,6852	0,7085	0,7199	0,6785	0,7014	0,6871	0,6712		
9 *Limaria hans*	0,7963	0,7291	0,6795	0,714	0,7178	0,7135	0,6323	0,2975	

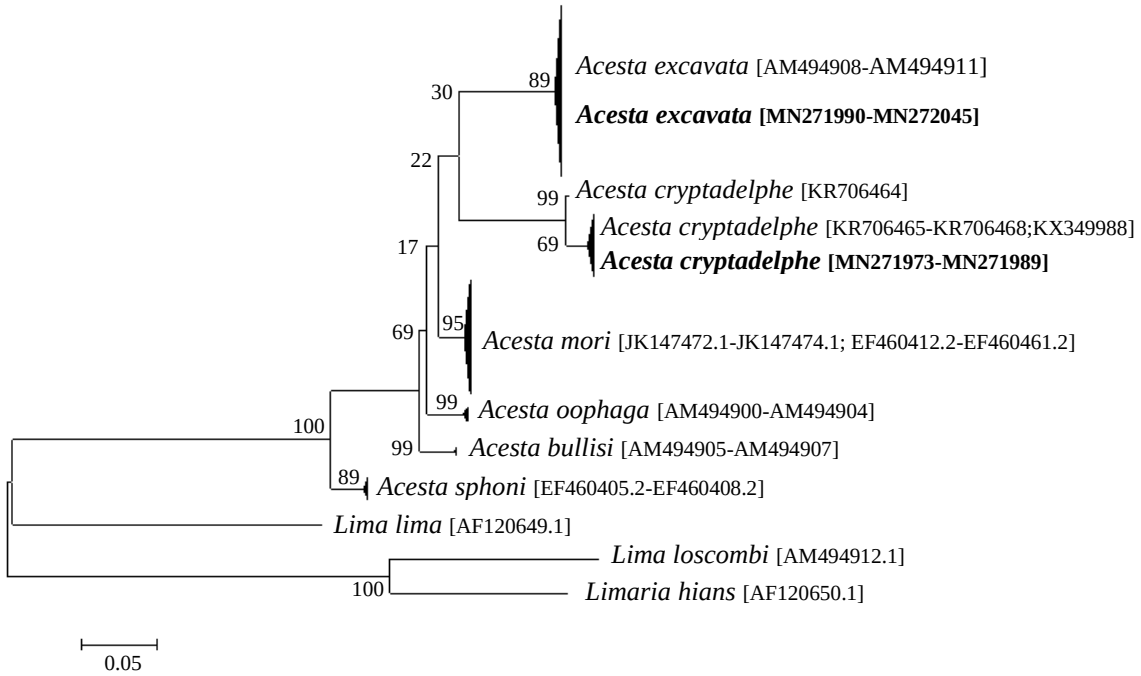

Figure 8 - Molecular phylogenetic analysis of *Acesta*, *Lima* and *Limaria* species by Maximum Likelihood method based on partial COI gene sequences (543 bp). Bootstrap values are shown next to the branches. Scale bar represents estimate sequence divergence.

3.2 Genetic diversity and population structure

A resumé of the genetic diversity of *Acesta excavata* and *A. criptadelphe* is presented in table 4, including sequences obtained from GenBank. Table 5 shows the distribution of the haplotypes among sampling sites.

Table 4 - Site-specific genetic diversity indexes for *Acesta excavata* and *A. cryptadelphe*. N: number of sequences; S: number of polymorphic sites; Eta: number of mutations; H: number of haplotypes; Hd: haplotype diversity; Var Hd: variance of haplotype diversity

Species	**Sampling site**	**N**	**S**	**Eta**	**H**	**Hd**	**Var Hd**	**Sd Hd**	**π**
Acesta excavata	LWest480	11	3	3	4	0.491	0.03078	0.175	0.00102
	AEast700	23	9	9	9	0.585	0.01493	0.122	0.0016
	AWest540	8	4	4	4	0.643	0.0339	0.184	0.00187
	AWest700	12	7	7	7	0.773	0.01628	0.128	0.00243
	Lis	2	0	0	1	0	0	0	0
	Norway	4	4	4	4	1	0.03125	0.177	0.00331
Acesta cryptadelphe	LWest1310	17	18	18	14	0.956	0.00190	0.044	0.00524
	NW Atlantic	6	18	18	4	0.8	0.02963	0.172	0.01138

Table 5 - Distribution of haplotype and haplotype frequencies per sampling site and haplotype total frequency of *Acesta excavata* and *A. cryptadelphe*.

Acesta excavata			
Haplotype	Sampling site	N. Ind/site	Total
Hap. 1	AWest540	5	35
	AWest700	6	
	AEast700	15	
	LWest480	8	
	Norway	1	
Hap. 2	AWest540	1	1
Hap. 3	AEast700	1	1
Hap. 4	AEast700	1	1
Hap. 5	AWest700	1	3
	AEast700	1	
	LWest480	1	
Hap. 6	AWest700	1	1
Hap. 7	LWest480	1	1
Hap. 8	AWest540	1	1
Hap. 9	AWest540	1	1
Hap. 10	AEast700	1	3
	Lis	2	
Hap. 11	AEast700	1	1
Hap. 12	AWest700	1	1
Hap. 13	AWest700	1	1
Hap. 14	AWest700	1	1
Hap. 15	LWest480	1	1
Hap. 16	Norway	1	1
Hap. 17	Norway	1	1
Hap. 18	Norway	1	1
Hap. 19	AEast700	1	1
Hap. 20	AEast700	1	1
Hap. 21	AEast700	1	1
Hap. 22	AWest700	1	1

Acesta cryptadelphe			
Haplotype	Sampling site	N. Ind/site	Total
Hap. 1	LWest1310	1	1
Hap. 2	LWest1310	4	4
Hap. 3	LWest1310	1	1
Hap. 4	LWest1310	1	1
Hap. 5	LWest1310	1	1
Hap. 6	LWest1310	1	1
Hap. 7	LWest1310	1	1
Hap. 8	LWest1310	1	1
Hap. 9	LWest1310	1	1
Hap. 10	NW Atlantic	1	1
Hap. 11	NW Atlantic	1	1
Hap. 12	NW Atlantic	3	3
Hap. 13	NW Atlantic	1	1
Hap. 14	LWest1310	1	1
Hap. 15	LWest1310	1	1
Hap. 16	LWest1310	1	1
Hap. 17	LWest1310	1	1
Hap. 18	LWest1310	1	1

3.2.1 *Acesta excavata*

The Acesta branch of the Whittard canyon shows higher haplotype diversity than the Lophelia branch; the two specimens collected in the Lisbon canyon share the same haplotype and each of the four sequences from Norway available in GenBank represent one haplotype. Overall, a total of 60 sequences of *Acesta excavata* revealed the existence of 22 haplotypes, generated by 24 mutations in 23 polymorphic sites. Of these 22 haplotypes, 3 are exclusive from Norway, 18 are only found in the Whittard canyon, 1 is shared between Norway and Whittard canyon and 1 between Lisbon with Whittard. All haplotypes except haplotypes 1, 5 and 10, were represented by only one individual (Fig.9 and Table 5). Haplotype 1 was the most frequent being shared by 34 individuals

from the Whittard canyon and one individual from Norway; Haplotype 5 was shared between three individuals from the Lophelia branch and Acesta East and West walls; Haplotype 10 (N=3) was the only haplotype found in the Lisbon canyon, but was also present in the east wall of the Acesta branch of the Whittard canyon.

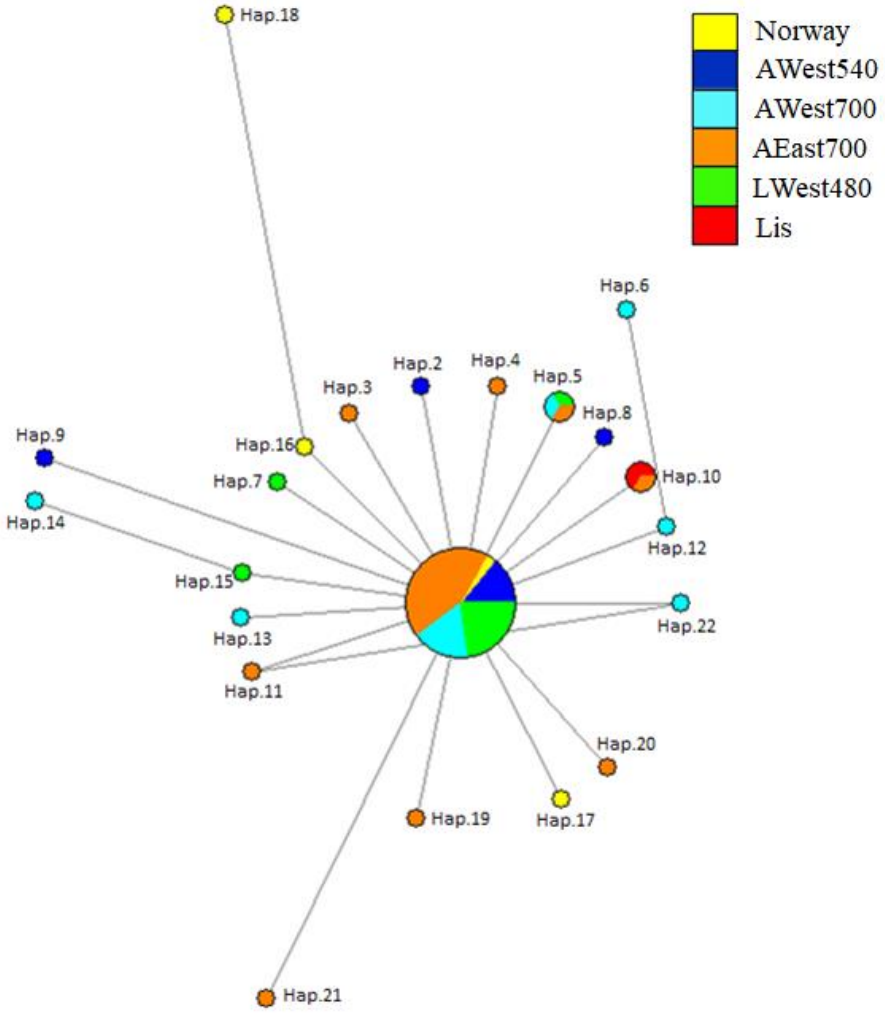

Figure 9 - Haplotype network using median-joining calculations for mtCOI gene of *Acesta excavata* from the Lisbon and Whittard canyons and from Norway. Node size is proportional to frequency.

Molecular variance (95% CI) was tested considering two different scenarios: *Scenario 1*: the samplings sites of Whittard canyon (LWest480, AWest540, AWest700 and AEast700); *Scenario 2*: the three sites along the European margin (Whittard, Norway, Lisbon). The neutrality of each sampling site was previously calculated in order to better perceive the robustness of the molecular variance test (Table 6).

Table 6 - Results of Tajima's D and Fu and Li's tests for neutrality for *Acesta excavata*. NS: not significant; S: statistical significant.

Sampling sites	Tajima's D neutrality test		Fu and Li's Netrality tests				
	Test val.	Stat. Sign.	D* test val.	Stat. Sign. (D*)	F* test val.	Stat. Sign. (F*)	Fs val.
LWest480	-1.87398	NS: 0.1 > P > 0.05	-1.87398	NS: 0.1 > P > 0.05	-2.03086	NS: P > 0.1	-2.042
AEast700	-2.249	S: P < 0.05	-3.47772	S: P < 0.02	-3.62311	S: P < 0.02	-7.589
AWest540	-1.7166	NS: 0.1 > P > 0.05	-1.66523	NS: P > 0.1	-1.79736	NS: P > 0.1	-1.236
AWest700	-1.71347	NS: 0.1 > P > 0.05	-1.85452	NS: P > 0.1	-2.06240	NS: 0.1 > P > 0.05	-4.027
Whittard canyon	-2.51871	S: P < 0.001	-4.74605	S: P < 0.02	-4.71037	S: P < 0.02	-23.700
Norway	-0.06501	NS: P > 0.1	-0.06501	NS: P > 0.1	-0.06004	NS: P > 0.1	-1741

AMOVA results for the Whittard canyon revealed a low value of genetic differentiation between the four sampling sites (Fst=0.00173), suggesting no differentiation. However, this value was not supported by the respective significance interval (p-value=0.41349+/-0.01556) (Table 7). For all sequences in 1 group (Table 8), Fst value indicates high genetic differentiation between samples, being sustained by a significant p-value (Fst=0.33752, p-value=0.0000+/-0.0000). Comparisons among groups of populations would be overestimated due to small individuals/group from Lisbon and Norway.

Table 7 - Amova results of LWest480, AWest540, AWest700 and AEast700 sampling sites of *Acesta excavata* from Whittard canyon. p-value<0.05

Source of variation	d.f	Sum of squares	Variance components	% of variation
Among populations	3	0.929	-0.00138 va	-0.29
Within populations	50	13.394	0.47835 vb	100.29
Total	53	14.323	0.47698	
Fixation Indexes		Fst:0.00173	p-value:0.41349+/-0.01556	

Table 8 - Amova results of all sampling sites of Acesta excavata from Whittard canyon, Lisbon canyon and Norway in one group. p-value<0.01*

Source of variation	d.f	Sum of squares	Variance components	% of variation
Among populations	2	3.565	0.23778 va	33.75
Within populations	57	26.602	0.46670 vb	66.25
Total	59	30.167	0.70448	
Fixation Indexes		Fst:0.33752	p-value:0.0000+/-0.0000*	

Pairwise genetic distances (Fst) and respective statistical significance were calculated between all locations of *Acesta excavata* (table 9). Significant p-values were obtained when the Whittard canyon was compared with each of the other locations.

Table 9 - Pairwise genetic distances of the sampling sites along the European margin Fst values presented below diagonal and respective p-values above it. In bold the significant values (p-value<0.02).

	Whittard canyon	**Lisbon**	**Norway**
Whittard canyon		**0.01758+-0.0037**	**0.00684+-0.0023**
Lisbon	0.46467		0.11816+-0.0097
Norway	0.25310	0.33758	

3.2.2 *Acesta cryptadelphe*

Whittard canyon shows a higher haplotype diversity of *Acesta cryptadelphe* than the Northwest Atlantic margin: in 17 specimens a total of 14 haplotypes were found in Whittard, whereas 4 haplotypes were identified from six individuals from the Northwest Atlantic, and no haplotype was shared between the two locations. Overall, the two locations combined registered 35 mutations in 34 polymorphic sites, originating 18 haplotypes (Table 5). Haplotype 2 was the most common in the Whittard canyon (4/17) and haplotype 12 the most abundant in the NW Atlantic (3/6). All the other 16 haplotypes were found only once with haplotype 11 (from Northwest Atlantic) having the highest differentiation (Fig.10 and Table 5).

The assessment of neutrality for the population of *Acesta cryptadelphe* from Whittard canyon showed statistical significance in the Tajima's D test, however, Fu and Li's tests did not indicate any deviation from the neutral evolution theory for any of the populations considered (Table 10). For this reason, AMOVA analyses could be performed. A very high level of genetic differentiation (Fst value: 0.85112, p-value:0.0000+-0.0000), with most variation occurring among populations, suggesting little genetic connectivity between both margins of the Atlantic (Table 11).

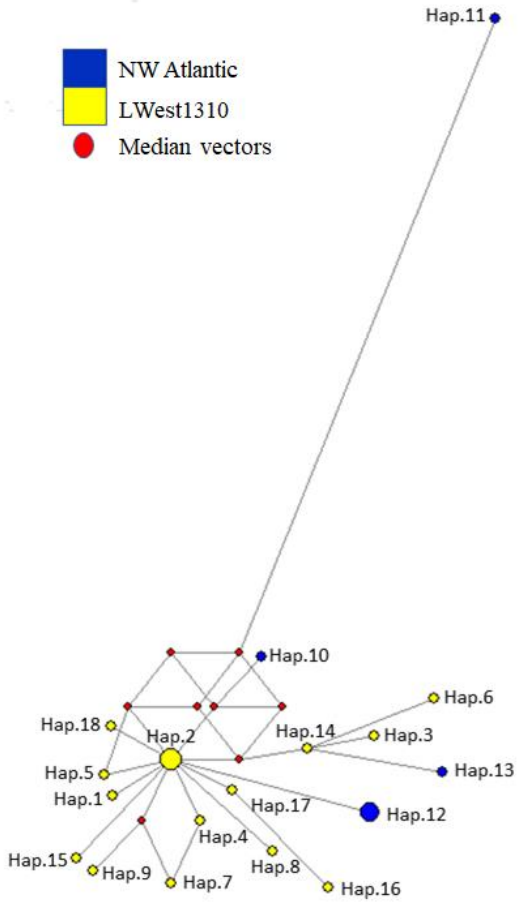

Figure 10 – Haplotype network using median-joining calculations for mtCOI gene of *Acesta cryptadelphe* populations. Node size is proportional to frequency.

Table 10 - Results of Tajima's D and Fu and Li's tests for neutrality for *Acesta cryptadelphe*. NS: not significant; S: statistical significant.

Populations	Tajima's D neutrality test		Fu and Li's Netrality tests				
	test val.	Stat. Sign.	D* test val.	Stat. Sign. (D)	F* test val.	Stat. Sign. (F)	Fs val.
LWest1310	-1.85252	S: P < 0.05	-2.00916	NS: 0.1 > P > 0.05	-2.27003	NS: 0.1 > P > 0.05	-11.028
NW Atlantic	-0.95976	NS: P > 0.1	-0.98859	NS: P > 0.1	-1.06930	NS: P > 0.1	1.739

Table 11 - Amova results of *Acesta cryptadelphe* populations from Whittard canyon and Northwest Atlantic in one group. p-value<0.01*

Source of variation	d.f	Sum of squares	Variance components	% of variation
Among populations	1	392.069	43.34896 va	85.11
Within populations	21	159.235	7.58263 vb	14.89
Total	22	551.304	50.93159	
Fixation Indexes		Fst:0.85112	p-value:0.0000+/-0.0000*	

4. Discussion

4.1 The first record of *Acesta cryptadelphe* in the Northeast Atlantic

In the marine environment, the difficulty of correctly identifying species is increased by their broad distribution and life stages occurring in the pelagic and benthic stages (Bucklin et al 2011). The traditional way of identifying marine species using solely their morphological traits begun to be considered unreliable since the past decades' advances in the molecular fields (Appeltans et al. 2012) In 2003, Paul Hebert suggested the creation of a database with sequences of the mitochondrial genetic marker cytochrome-c-oxidase I gene as a template for identification of specimens, which was later developed and designated as DNA barcoding (Hebert et al. 2003; Savolainen et al. 2005). Barcoding allows the assessment of genetic variation within and between populations or species, the reconstruction of evolutionary relationships and inference processes of speciation even for the smallest and rare organism (Bucklin et al. 2011; Sunnucks 2000), regardless of its life stage (Bucklin et al. 2011). Additionally, barcoding revealed the occurrence of cryptic species: morphologically identical but genetically different (Trivedi et al. 2016).

This study reports for the first time the presence of *Acesta cryptadelphe* in the Northeast Atlantic. Up to now *A. cryptadelphe* was only known from the Norwest Atlantic margin (off Newfoundland and Nova Scotia), where it was found associated with rocky substrates below 400 m water depth, either on isolated outcrops, under overhangs or on rock walls (Gagnon et al. 2015). Firstly identified as *A. excavata* (Gagnon and Haedrich 2003), the use of molecular tools associated to traditional morphological measurements allowed these authors to describe *A. criptadelphe* as cryptic to *A. excavata* (Gagnon et al. 2015).

Herein, the use of molecular methods allowed, once again differentiating between these two species, highlighting the importance of such methods in the difficult task of identifying the 100s of thousands of species that are expected to occur in the deep sea (Appeltans et al. 2012 and references within) and supporting the expectations of increasing discoveries when using new technologies (Witt et al. 2006), including precise sampling with ROV's (Danovaro et al. 2014). In previous studies of community composition in the Whittard canyon, only *Acesta excavata* was reported (e.g. Huvenne et al. 2011, Huvenne et al. 2016 and Johnson et al. 2003) leading to the initial hypothesis that all the sampled individuals from this dataset would belong to this species. The discovery of *A. cryptadelphe* in the Whittard canyon provide a good illustration of the taxonomic issues that still persist in deep-sea ecosystems, even in the most well-known species of some of the most-well known habitats.

In the Whittard canyon, *Acesta cryptadelphe* was collected from walls at about 1300 m with no other occurrences in shallower waters where *A. excavata* was found. This bathymetric segregation

between species suggests that *A. cryptadelphe* can explore different environmental conditions that may explain the occurrence of possibly competing species inside the canyon. Gagnon et al. (2015) suggested that this misidentification could also have occurred in individuals from the Mid-Atlantic Ridge (north of the Azores). However, the water depth at which these individuals were sampled is not present and it is therefore impossible to infer an association between the distribution of *A. cryptadelphe* and specific abiotic conditions. The recognition of cryptic species with different niches is essential for planning and implement effective conservation measures of those particular species, as two species, instead of one should be considered, but also of the habitats they occur, in this case cold-water corals.

With the discovery of *Acesta cryptadelphe* in the Whittard canyon new questions arose during this study, namely if contemporary connectivity occurs between the two margins of the Atlantic. The results obtained suggest that the populations of *A. cryptadelphe* from both sides of the Atlantic are not currently connected. This is supported by the "star-shaped" haplotype network with a central node (Hap. 2, from the Whittard canyon) from where all other haplotypes radiate. With the exception of this central haplotype and Hap. 12 (from northwest Atlantic), all other haplotypes occurred in a single individual. In order to connect the haplotypes with maximum parsimony within the Whittard canyon and the Northwest margin, medium vector calculations had to be performed which suggests the existence of several haplotypes that were not sampled. It is possible that these missing haplotypes are a consequence of a low sampling effort in both sides of the Atlantic, or may represent populations in between both margins, namely in the Mid-Atlantic Ridge. However, the extremely high value of genetic differentiation (Fst: 0.08511) that attributes more than 85% of variation in the haplotype sequences to differences among the two populations suggests that connectivity between these populations is practically inexistent, with no gene flow across the Atlantic Ocean.

4.2 Population structure and connectivity of *Acesta excavata* in the NE Atlantic

Spatial planning is gaining attention in the context of deep-sea conservation, but the data to support decisions are scarce, particularly data on population connectivity, which is critical both for the design of marine reserve networks to protect biodiversity and for the development of strategies to protect species associated with degrading seascapes. *Acesta excavata* is one of the most common species associated with CWC in the European margin, in both reef formations and vertical walls of submarine canyons (Järnegren and Altin 2006; López Correa et al. 2005). Similar to the CWC species to which it is associated, *A. excavata* has a long lifespan and slow growth and is therefore extremely

vulnerable to habitat damage. The Whittard canyon is currently not included in any protected area but the presence of vulnerable marine ecosystems, namely aggregates of *Lophelia pertusa* and associated *Acesta excatata* (and *A. cryptadelphe*) make it a good candidate for protection, including its inclusion in a network of MPA's extending the conservation efforts already applied in Rockall Trough, Darwin Mounds and Porcupine Seabight, all suitable habitats for *Acesta* spp. and from where *Lophelia pertusa* is reported (Freiwald et al. 2014).

To my knowledge, this study is the first attempt to assess genetic population structure and connectivity between populations of *A. excavata* along the European margin and within a complex hydrodynamic system, such as the Whittard canyon. However, the results obtained in this study must be carefully interpreted because of the differences in the number of DNA sequences available for each of the studied sites, which may lead to an underestimation or overestimation of haplotype diversity, this is particularly obvious in the sequences of Norway, from which each of the four available sequences represented on haplotype, yielding a haplotype diversity (Hd) of 1, and for the Lisbon canyon, for which the two obtained sequences belonged to the same haplotype. Additionally, the east side of the Acesta branch of the Whittard canyon (AEast) presented a deviation from the neutral evolution theory, indicating that gene flow and genetic drift are not the only factors influencing genetic structure on this site and future studies are needed to understand the role of other differentiation mechanisms. Nevertheless, some of the results are well grounded and worth discussion.

The haplotype network has a well-defined "star-shaped" configuration, typical of populations under expansion, with Hap.1 as the central node from where all other haplotypes segregate without apparent partitioning according to sampling site. Inside the Whittard canyon, despite the occurrences of many singletons, the Fst value obtained was close to zero, indicating that the differentiation between the sampling sites is practically null and that those differences are due to variation within the sites rather than between sites. These results suggest that the population of *Acesta excavata* in the Whittard canyon is not structured and that genetic connectivity occurs between the sampling sites of the two branches. Haplotype diversity does not seem to be correlated to sample size, as LWest480 (N=11) present lower diversity than all sampling sites of the Acesta branch, where AWest540 (N=8) and AWest700 (N=12) presented Hd values of 0.643 and 0.773, respectively, fitting the range of those recorded for *A. mo*ri and *A. sphoni* by Clague et al. 2011. These differences may be explained by differences in the abiotic conditions of the different branches. Indeed, the southern branch, designated as Lophelia branch because the coral *Lophelia pertusa* covers 70% of the walls (Huvenne et al. 2011), with only small agglomerations of *A. excavata* individuals (Amaro et al. 2016), whereas the northern branch, the Acesta branch is dominated by *A. excavata* in association with *Neopycnodonte zibrowii* was reported, with no reports of *L. pertusa* (Johnson et al.

2013; Huvenne et al. 2011). This preference for the northern branch may indicate ideal conditions for settlement, promoting diversity, whereas the settlement in less suitable conditions may only be possible to more "adapted" haplotypes.

As already mentioned, because of the small number of samples from the Lisbon canyon and Norway, the assessment of genetic connectivity along the European margin is greatly conditioned and therefore the results of this thesis can only provide a hypothesis that needs to be addressed in the future. Despite the great distances that separate the three studied locations, Norway and the Lisbon canyon share one haplotype each with the Whittard canyon suggesting a shared ancient polymorphism or present connectivity between locations. In the latter case, the obtained fixation index (Fst=0.33752) indicates high genetic differentiation, but only 33.75% of with explained by differences between the three locations. Differentiation across sites is supported by significant differences in Fst distances between the Whittard canyon and each of the other two locations, suggesting low gene flow along the European margin. Nevertheless, because of the presence of shared haplotypes it is not possible to discard the hypotheses of connectivity between populations of *Acesta excavata* in the NE Atlantic. Future investigations must consider the existence of other populations in intermediate habitats that allow genetic exchange among them, creating a string of gene flow between farther apart populations, i.e. following a stepping-stone model.

Gene flow between populations of *Acesta excavata* depends on the dispersal of larvae by ocean currents. One possible means of transportation, connecting northern and southern populations along the European margin is the branch of the superficial North Atlantic current (NAC) (Fig.11), that enters through the Porcupine Bank and flows across the Bay of Biscay towards the coast of Spain, responsible for a southward movement of the superficial water masses (Pingree and Garcia-Soto 2014). Another possibility, in the opposite direction, is the transport through the Mediterranean Outflow Water (MOW), which passes the West Iberian Margin up to 1500 m deep (Stigter et al. 2011), including the Lisbon canyon and continues north, entering the Bay of Biscay and the Whittard canyon at approximately 1200 m deep (Fig.11). Continuing northward, the Eastern North Atlantic Water (ENAW) is a shelf edge current that mixes with the MOW and latter joins the branch of the North Atlantic Current (NAC) that feeds the Nordic Sea (New et al. 2001) and could possibly secure connectivity with populations from Norway (Fig.11).

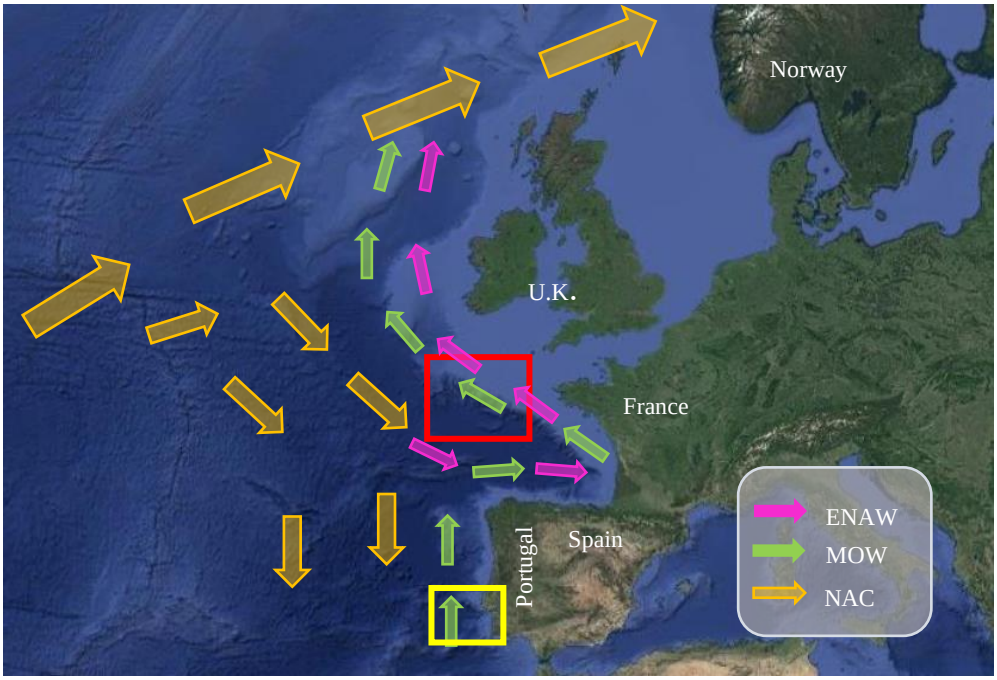

Figure 11 - Schematic representation of the oceanic current circulation along the Northeast European margin. The yellow square marks the Lisbon canyon in the Western Iberian Margin, the red square identifies the Whittard canyon in the Bay of Biscay. NAC: North Atlantic Current; ENAW: Eastern North Atlantic Current; MOW: Mediterranean Outflow of Water.

One possible to test these hypotheses would be through the application of coupled biophysical models, incorporating ocean circulation and biological traits, particularly reproductive and larval development (Werner et al. 2007). *Acesta excavata* has a semi-continuous reproductive cycle with females broadcasting buoyant eggs of 160 μm. Both the size of the larval shell and the size of the spawned eggs are suggestive of pelagic lecithotrophic development (Jarnegren et al. 2007). Nevertheless, as commonly occurs in deep-sea species (Hilário et al. 2015), knowledge on the pelagic larval duration (PLD) is still missing, which is crucial to infer the maximum geographic dispersal distance of the species and resolve the possible connectivity patterns hypothesised above.

5. Conclusion and Future Investigations

The main result of this study: the discovery of *Acesta cryptadelphe* in the Northeast Atlantic was only possible with the application of molecular methods that allow to overcome the difficulties of morphological identification, especially in the case of cryptic species living in the same habitat and geographical region. This new report supports the idea that, despite the many progresses made in the last decades, an accurate account of the biodiversity in the deep-sea is far from being obtained, and that previous identifications should be considered as a current hypothesis, open to reassessment when necessary, like any other scientific result.

The Whittard canyon is now known to host two representatives of the genus *Acesta*, *A. excavata* and *A. cryptadelphe*, occurring in areas of the reef forming coral *Lophelia pertusa*. Whistle it was not possible to infer connectivity patterns between populations of *A. cryptadelphe*, as only one population is known in the NE Atlantic, it was possible to assess that the persistence of this population does not depend on migration processes across the Atlantic. Exploration of other locations with suitable habitat for the settlement of *Acesta*, and reassessment of the populations so far assumed to be of *A. excavata* are essential to determine the extent of the distribution of *A. cryptadelphe* in the NE Atlantic and Mid-Atlantic Ridge, and for future studies of connectivity of this species between both sides of the Atlantic ocean and possibly along the European margin.

Regarding *A. excavata*, this study concluded that gene flow does occur within the Whittard canyon and that no apparent barriers to dispersal exist between the branches of this canyon. However, it was not possible to determine connectivity, or its absence, along the European margin. The lack of answers is intimately related with the small number of individuals from Norway and Lisbon canyon as well as of sampling sites. Nevertheless, it is possible to suggest that population of *A. excavata* are connected, not across such great distances as between the Iberian and Norwegian margins, but between more closely located populations. These results, although not conclusive allow to set new hypotheses that can be tested in the future.

Realistic insights of population structure cannot rely solely in genetic data. Demographic studies such as birth and mortality rates, size of populations, self-recruitment and migration rates, provide information for inferences of connectivity. Additionally, for organisms with a pelagic larval stage, knowledge about spawning intervals, origin of larvae, mortality rates, PLD and transport distances, conjugated with local hydrology, becomes essential to understand dispersal patterns. The application of biophysical models and the use of natural markers such as geochemical tags, naturally incorporated in calcified structures or introduction of stable isotopes artificially are becoming increasingly frequent techniques to comprehend the processes of spatial and temporal connectivity

(Cowen and Sponaugle 2009; Corre et al. 2012) all of which are possible to apply to study connectivity between populations of *Acesta*.

The use of an integrative approach will be fundamental to understand the scale of connectivity between populations of *Acesta excavata, and A. cryptadelphe,* and to determine the biological and physical processes underlying the observed connectivity patterns. This knowledge is in turn crucial to evaluate the resilience of populations to human impacts and to defining spatial management strategies, including marine protected areas that are effective in the protection of spatially fragmented populations, such as those of CWC and their associated fauna.

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Appendix I

Material and Methods

Appendix I – GenBank Sequences

Suplementary Table 1 - GenBank sequences details: species identification, number of sequences (N), collection sites, research article containing sampling information and study for each species and respective accession number

Species	**N**	**Collection Site**	**Reference**	**Acession Number**
Acesta bullisi	3	Gulf of Mexico	Järnegren et al. 2007	AM494905; AM494906; AM494907
Acesta oophaga	5	Gulf of Mexico	Järnegren et al. 2007	AM494900; AM494901; AM494902; AM494903; AM494904
Acesta excavata	4	Norway	Järnegren et al. 2007	AM494908; AM494909; AM494910; AM494911
Acesta sphoni	8	Eastern Pacific margin	Clague et al. 2011	EF460405.2; EF460406.2; EF460407.2; EF460408.2; JK147468.1; JK147469.1; JK147470.1; JK147471.1
Acesta cryptadelphe	6	NW Atlantic (Newfoundland; Nova Scotia; Norfolk Canyon)	Gagnon et al. 2015	KX349988.1; KR706464.1; KR706465.1; KR706466.1; KR706467.1; KR706468.1
Acesta mori	40	Eastern Pacific margin	Clague et al. 2011; Walz et al. 2014	EF460412.2; EF460413.2; EF460415.2; EF460416.2; EF460417.1; EF460418.1; EF460420.2; EF460421.1; EF460423.1; EF460424.1; EF460425.1; EF460426.2; EF460427.2; EF460428.2; EF460429.1; EF460431.2; EF460432.2; EF460434.1; EF460435.1; EF460436.1; EF460438.2; EF460439.2; EF460442.1; EF460443.2; EF460444.2; EF460446.2; EF460447.2; EF460448.2; EF460449.2; EF460450.2; EF460451.2; EF460453.2; EF460454.2; EF460456.2; EF460457.2; EF460459.2; EF460461.2; JK147472.1; JK147473.1; JK147474.1
Limaria loscombi	1		Järnegren et al. 2007	AM494912.1
Lima lima	1		Giribert and Wheeler 2002*	AF120649.1
Limaria hians	1		Giribert and Wheeler 2002*	AF120650.1

*Giribert G. and Wheeler W.C. (2002). "On Bivalve Phylogeny: A High-level Analysis of the Bivalvia (Mollusca) based on Combined Morphology and DNA Sequence Data." *Invertebrate Biology* 121:271-324.