

UNIVERSIDADE DO ALGARVE

GENETIC CONNECTIVITY PATTERNS IN HOLOTHURIA
MAMMATA CONSIDERING DIFFERENT SPATIAL SCALES

Filipe Freitas Henriques

Dissertação para obtenção do grau de:

Mestrado em Biologia Marinha

Trabalho efetuado sob a orientação de:

Mercedes González- Wangüemert, PhD

Ester A. Serrão, PhD

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II. ABSTRACT

As a consequence of the world population growth, the demand for sea food resources is increasing. Consequently, worldwide fisheries transition from depleted finfish resources to alternative invertebrates species created a massive industry. Sea cucumber stocks have been overfished in Indo-Pacific Oceans as result of lack of effective management, non-regulated fisheries and an increasing demand from Oriental countries. The *õbeche de merõ* demands have induced expansion of their fisheries worldwide and have resulted in catches of new target species from the Mediterranean Sea and NE Atlantic Ocean. With high commercial value and fragile life-history traits, sea cucumbers are particularly vulnerable to commercial fisheries, especially when there are no scientific knowledge to support their effective management.

This study aims to fill in some of the gap in scientific data about sea cucumber populations, namely *Holothuria mammata*. It was assessed the genetic diversity and structure, connectivity and effective population size with novel polymorphic molecular markers (microsatellites). This analysis was done in several spatial scales and considering oceanographic patterns. Some morphometric traits were also analysed, such as the distribution of length and weight classes.

The results showed that *Holothuria mammata* has globally high genetic diversity, higher genetic connectivity between Atlantic populations and genetic differentiation between Atlantic/Mediterranean and eastern/western Mediterranean basins. Effective population sizes were smaller in the Atlantic, showing some mutation-drift disequilibrium. Oceanographic patterns were strongly correlated with the genetic differentiation patterns. Atlantic populations presented bigger individuals (*i.e.* length and weight) than Mediterranean ones, clearly associated with environmental conditions, and ecology features. Biometric data and genetic analysis allowed us to establish the three potential stocks inhabiting the geographic area and improve the biological knowledge of this new target species. This information will be useful to suggest the first recommendations to its effective fishery management, and future comparisons in order to assess the fishery effects either at genetic and/or morphometric level.

Key Words: *Holothuria mammata*, genetic structure, oceanographic patterns, microsatellites, morphometry, fishery management.

III. RESUMO

Com a população Mundial na ordem dos 7,2 bilhões e projeções a apontar para um aumento populacional na ordem dos 9.6 bilhões para 2050, é previsto um aumento a nível Mundial pela procura dos recursos alimentares marinhos. Apesar de uma recente estabilização da quantidade de capturas de pescado, estima-se que pelo menos, 28.8% dos stocks pesqueiros globais estejam sobreexplorados. A situação poderá ser potencialmente preocupante, se tivermos em conta que o atual nível de capturas apenas se tem mantido relativamente constante devido a uma persistente procura por novos recursos pesqueiros e pela expansão das áreas de pesca. Como consequência do aumento da procura internacional por recursos pesqueiros, existe evidências muito claras que o nível trófico médio das espécies alvo está a diminuir, significando uma transição nas descargas de pescado, de espécies de nível trófico alto (*e.g.* grandes predadores), para níveis tróficos baixos (*e.g.* invertebrados e peixes planctívoros). Esta situação resulta de um declínio Mundial dos predadores de topo e conseqüentemente, um aumento da pressão pesqueira em outros recursos pesqueiros, como os invertebrados, na qual os moluscos, crustáceos e equinodermes representam os mais afetados com o aumento da demanda global. Além dos impactes ambientais negativos originados pela pesca (*e.g.* arrasto de fundo, by-catch, rejeições, sobrepesca, entre outros) também são registadas alterações na biologia e ecologia dos organismos marinhos, desde variações na diversidade genética e estrutura das populações, a modificações na estrutura e função dos ecossistemas marinhos. A sobre-exploração promove a perda de diversidade genética, através da redução do tamanho efetivo da população (N_E), e conseqüentemente uma diminuição da variação genética e fenotípica, tal como o tamanho corporal, fecundidade, fitness e taxa de crescimento. Estes efeitos têm-se mostrado não reversíveis mesmo após a libertação da pressão pesqueira. A implementação de uma gestão pesqueira sustentável permite manter a diversidade genética das populações e o seu potencial a nível evolutivo, mas também a resiliência dos ecossistemas.

A falta de gestão efetiva das pescas e/ou políticas de conservação, são das principais causas para a sobrepesca. Na maioria dos casos tal deve-se à falta de conhecimento ecológico e/ou biológico das espécies comerciais. Este tipo de situações acontece repetidamente quando se inicia a exploração de novos recursos pesqueiros.

Os stocks de pepinos de mar nos Oceanos Indo-Pacífico têm sido um exemplo em que a sobre-exploração ocorreu não só pela falta de conhecimento

ecológico/biológico, mas também devido a pescas não regulamentadas. Com os stocks asiáticos praticamente esgotados, e sendo a procura por este recurso pesqueiro cada vez maior, a forma mais fácil de responder a esta procura, foi expandir a pesca de pepinos do mar a um nível global, na qual inclui o Mar Mediterrâneo e Oceano Atlântico. Com alto valor comercial e histórias de vida frágeis, os pepinos do mar estão particularmente vulneráveis á pesca comercial, principalmente quando não existe estudos biológicos, ecológico ou genéticos que possam aconselhar e suportar a sua gestão sustentável.

Estudos recentes têm demonstrado o potencial farmacológico das holotúrias, com descobertas de compostos com propriedades antimicrobianas, antioxidantes e anticancerígenas.

Os pepinos do mar são espécies igualmente importantes para o funcionamento saudável dos ecossistemas, pois para além de decomporem a matéria orgânica contida nos sedimentos, reciclam os nutrientes, estimulando o crescimento de microalgas e prevenindo eutrofização dos fundos marinhos.

Consideramos urgente iniciar estes estudos de modo a averiguar os parâmetros biológicos e ecológicos fundamentais para dar início a uma gestão de pescas regulamentada e efetiva. Informações como taxas de crescimento e mortalidade, longevidade, biologia reprodutiva, duração larvar, diversidade genética, padrões de conectividade e estrutura genética, permitiram implementar uma gestão pesqueira sustentável a médio e longo prazo. No âmbito desta necessidade de fornecimento de informação sobre os pepinos do mar, este estudo analisou e avaliou a diversidade genética e respetiva estrutura genética populacional, a sua conectividade e o tamanho efetivo das populações. Para tal foi feita uma recolha de amostras de 8 populações ao longo do Mar Mediterrâneo e do Oceano Atlântico, cobrindo grande parte da distribuição geográfica da espécie alvo de estudo, a *Holothuria mammata*. Desenvolveu-se também marcadores moleculares específicos para esta espécie (*i.e.* Microsatélites). A análise genética foi feita em varias escalas espaciais e tendo em conta os padrões oceanográficos das correntes de superfície, de forma a compreender como se processa a dispersão larvar de acordo com a conectividade genética.

Para além das análises genéticas, também foi analisado algumas características morfométricas como a distribuição de classes de comprimento e peso.

Os resultados deste estudo mostraram que a *Holothuria mammata* apresenta a nível geral, grande diversidade genética, uma maior conectividade genética entre as populações do Atlântico e diferenciação genética entre Atlântico/Mediterrâneo como

também entre as bacias este e oeste do Mediterrâneo. As populações do Oceano Atlântico apresentaram evidências de um desequilíbrio entre a mutação e a deriva genética. Também verificou-se que o tamanho efetivo das populações do oceano Atlântico são menores do que as populações do Mar Mediterrâneo.

Os padrões oceanográficos das correntes de superfície demonstraram estar fortemente correlacionados com os padrões de diferenciação genética encontrados.

A nível das análises morfométricas efetuadas, verificou-se que a *Holothuria mammata* apresenta uma morfometria alométrica, evidenciando uma alteração na forma corporal á medida que o animal cresce. As populações do oceano Atlântico apresentaram indivíduos maiores, quer a nível do comprimento como a nível do peso, do que as populações do mar Mediterrâneo. Estas diferenças corporais estão associadas às diferentes condições ambientais, tal como a disponibilidade de habitat e alimento, e também às características ecológicas, como a competição por alimento.

As análises biométricas e genéticas, permitiram-nos identificar os stocks existentes na área geográfica do estudo e contribuir para o conhecimento da biologia desta nova espécie com interesse comercial. Pelo menos três stocks devem ser considerados; um stock para as populações do Atlântico, e dois stocks para o Mar Mediterraneo (Bacias Este e Oeste).

A informação contida nesta Tese, terá grande utilidade na sugestão das primeiras recomendações para uma gestão efetiva das pescas. Adicionalmente, estes dados irão servir para futuras comparações quer a nível genético, como a nível morfométrico, de modo a avaliar os efeitos da pescaria.

Palavras-chave: *Holothuria mammata*, estrutura genética, padrões oceanográficos, microssatélites, gestão de pescas.

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1. Introduction

1.1. State of the World Fisheries

Oceans, covering nearly 71% of Earth surface, are the oldest reservoir of biodiversity, being our main life support system. The goods and services provided from marine biodiversity are harboured on a huge and still increasing list, being the human the main beneficent (Roff & Zacharias, 2011). Earth's population suffered an important growth during the last centuries, from about 600 million people in 1700 to 6.3 billion in 2003 (Cohen, 2003). According recent predictions, the current 7.2 billion is projected to increase by almost one billion people in the next eleven years, reaching 9.6 billion in 2050 (United Nations, Department of Economic and Social Affairs, 2013). As consequence of this population growth, the demand for food is also increasing specially from marine resources.

Despite apparent stabilization of overall global capture fisheries (at 90 million tonnes; Figure-1), the percentage of overexploited stocks has exhibited a general increasing trend in the last decades (32,5 % of global stocks in 2008 were overexploited), although more recent data shows a decreasing of overexploitation to 28,8% in 2011 (FAO, 2014) . In this thesis I will use the *stock* definition provided by Begg *et al.* (1999): *stock* describes characteristics of semi-discrete groups of fish/invertebrates with some definable attributes which are of interest to fishery managers

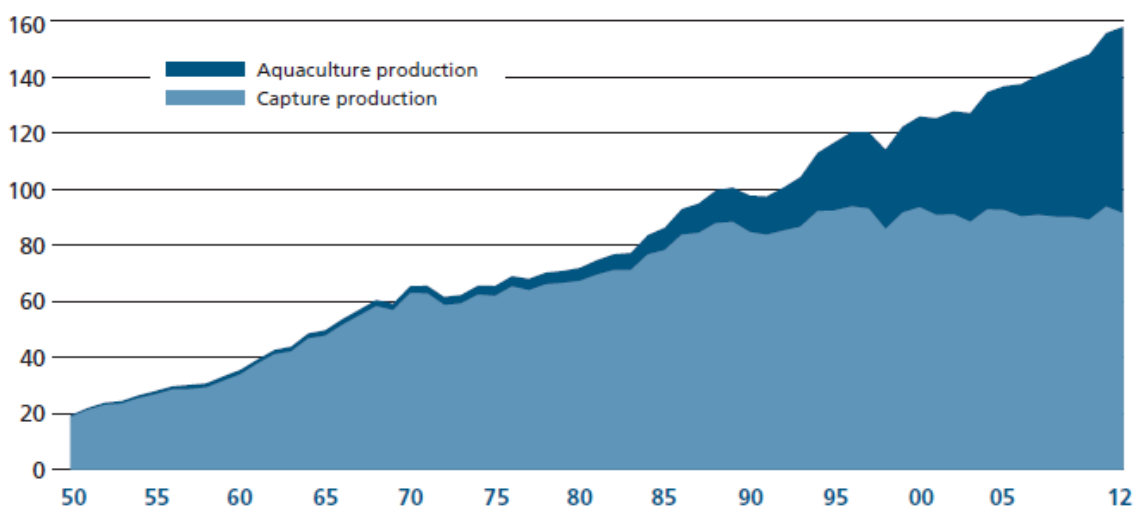


Figure 1: World capture fisheries and aquaculture production (Million tonnes) between 1950 and 2012. Source: adapted from (FAO, 2014)

Overfishing not only causes negative ecological consequences, it also reduces fish/invertebrate production, leading to adverse social and economic consequences. It is estimated that rebuilding overfished stocks could increase fishery production by 16.5 million tonnes and annual rent by \$32 billion, which would increase the food security, economies and well-being of the coastal communities (FAO, 2014), especially in under-development countries, where marine food resources are a vital source of protein and essential nutrients.

From a global perspective, there is strong evidence that the trophic level of target species are decreasing, meaning a transition in landings from high (big predators) to low trophic levels (invertebrates and planktivorous pelagic fish). This situation is causing global decline of top predators and consequently, an increasing pressure shifted to non-fish resources (Pauly, 1998). In fact since 1950 until present, invertebrates total global catch has been increasing from 2 to 12 million tonnes; nevertheless, this expansion has only been maintained due to new target species as cephalopods and echinoderms and a higher number of countries fishing invertebrates (Anderson *et al.*, 2011a; Eriksson *et al.*, 2012).

1.2. Genetics and Fisheries Management

Extensive evidence exists indicating the direct impacts of fishing on marine biodiversity, but also the collateral ones such as bottom trawling (*e.g.* Jones, 1992), by-catch (*e.g.* Hall *et al.*, 2000) and discards (*e.g.* Harrington *et al.*, 2005). However, only recently its being recognized that fishing has also effects on the biology and ecology of marine organisms, from changes on genetic diversity and structure of populations, to modifications on the structure and function of marine ecosystems (Pope *et al.*, 2000; Stokes & Law, 2000; Scheffer & Carpenter, 2003; Stockwell *et al.*, 2003; Kuparinen & Merila, 2007; Roff & Zacharias, 2011; Allendorf *et al.*, 2014).

With the development of biochemical and molecular techniques, the discipline of population genetics has evolved from a theoretic field to a powerful empirical approach (Charlesworth & Charlesworth, 2009; Allendorf *et al.*, 2013) . There are some subjects which can be address through the application of population genetics to fisheries management and to conservation (Ryman & Utter, 1987; Ward, 2000; Abaunza *et al.*, 2008; Kochzius *et al.*, 2010; Abdul-Muneer, 2014).

1.2.1. Genetic Diversity and Effective Population Size

Genetic diversity, is a useful parameter to assess the health or fitness of target species. In fact, the genetic diversity is recognized by the International Union for the Conservation of Nature (IUCN) like one of the three forms of biodiversity, that needs to be conserved (Roff & Zacharias, 2011) in order to maintain the evolutionary potential of species and populations (Allendorf *et al.*, 2013). Overexploitation promotes the loss of genetic diversity (Allendorf *et al.*, 2014; Pinsky & Palumbi, 2014), either by reducing population size directly and/or decreasing the number of migrants between local populations (Hindar *et al.*, 2004; Allendorf *et al.*, 2013). Nevertheless, the number of reproductively successful individuals, defined in population genetics as the effective population size or \bar{N}_e (Watts *et al.*, 2007), determines the level of inbreeding and the rate of loss of genetic diversity. When a species suffers strong reductions of its effective population size (*e.g.* by overfishing), the stochastic effect of genetic drift increases, unbalancing the drift-mutation equilibrium, in which the rate of mutation (*i.e.* source of diversity) is not enough to balance the loss of diversity determined by the genetic drift (Hare *et al.*, 2011; Allendorf *et al.*, 2014; Pinsky & Palumbi, 2014). Moreover, as N_e declines, the balance between natural selection and genetic drift is also altered, favouring changes in gene frequency (Hare *et al.*, 2011). A reduced N_e could also erode the genetic variation by increasing the probability of fixation of deleterious alleles, instead of fixation of adaptive alleles chosen by selection, reducing overall fitness and limiting adaptive responses to environmental change (Allendorf *et al.*, 2013). On the other hand, the use of historical N_e could be very useful to the fisheries management because the pre-exploitation estimates of abundance can be corrected, considering that demographic data from fisheries resources are normally collected after the onset of exploitation (Ovenden *et al.*, 2013).

1.2.2. Population Genetic Structure

Most marine species are subdivided (or structured) in time and space by several local random mating units, or subpopulations (Roff & Zacharias, 2011; Allendorf *et al.*, 2013). In fisheries management, these demographically cohesive groups of individuals are considered as stocks, although many other definitions have been provided until now (Çiftci, 2002; Ovenden *et al.*, 2013 and references therein). Knowledge of populations

structure is critical for the understanding the biology of the species (Gharbi & Said, 2011) and especially important to their fishery management because of the delimitation of stocks could optimize the overall productivity and protect the most vulnerable populations (ICES, 2014). Moreover, preserving the population structure and the evolutionary processes that lead to structuring among populations (*i.e.* drift, selection, migration and mutation), will guarantee the range of diversity within a species over a variety of environmental conditions, and therefore enhance the natural resilience to perturbations (Eldridge *et al.*, 2009).

1.2.3. Connectivity

Genetic connectivity or gene flow is the number of migrants interchanged between populations that successfully contribute to gene pool (Hedgecock *et al.*, 2007a). Gene flow is an evolutionary force that reduces genetic differences between populations and increases variation within populations. Understanding population connectivity is an essential pre-requisite for implementing effective fishery management, either in delineation of appropriate scales for management as well in the development of networks of marine protected areas (MPAs) (Fogarty *et al.*, 2007). A right design of MPAs network will maximize larval export and spillover of adults to adjacent fishing areas, allowing higher yields and profits, while maintaining self-persistence (*i.e.* inter-reserve connectivity) of their subpopulations (Halpern, 2003; White *et al.*, 2008; Gaines *et al.*, 2010).

1.2.4. Selection

Overharvesting promotes unnatural selection (*i.e.* fisheries), through removal of phenotypes most chosen by natural and sexual selection, and leaving survivors with relatively high fitness to the fishing selection, but less optimal with respect to natural selection, jeopardizing the evolutionary potential of species (Thériault *et al.*, 2008; Allendorf *et al.*, 2013). In fish for example, about 20-30% of the variation in life-history traits, such as age and size at maturation, is heritable (Stokes & Law, 2000; Law, 2007), therefore the effects of harvesting on individual life histories should receive more attention.

Several examples have been published showing the fishery effects on the exploited stocks: reduced body size (Grift *et al.*, 2003; Olsen *et al.*, 2004; Consuegra *et al.*, 2005; Swain *et al.*, 2007), earlier age at maturation (Grift *et al.*, 2003; Olsen *et al.*, 2004; Swain *et al.*, 2007), slower growth rate (Swain *et al.*, 2007), earlier time of spawning (Consuegra *et al.*, 2005), and low fecundity (Hutchings & Baum, 2005; Jørgensen *et al.*, 2007; Biro & Post, 2008). Therefore, fisheries are an important source of selective pressures favouring particular life histories, morphologies and behaviours. Morphometric data such as length and weight, although being considered basic, can be useful for morphological and life historical comparisons between stocks at several spatial scales (Froese, 2006). These baseline data could help on the implementation of effective management and conservation policies, mainly when ecological and biological information from the new target species is lost, such as on European sea cucumbers (González-Wangüemert *et al.*, 2014a).

Recent studies have also demonstrated that fisheries reduce the genetic diversity of the populations (Pérez-Ruzafa *et al.*, 2006; Pinsky & Palumbi, 2014; González-Wangüemert *et al.*, 2015). Genetic changes due to fisheries cannot be avoided in most cases, but they should be detected and measured their magnitude (Allendorf *et al.*, 2014). Therefore, the genetic monitoring can reveal a comprehensive picture of the target population status, by assessing both ecological and evolutionary time frames, being this methodology more reliable and cheaper than traditional monitoring approaches (Schwartz *et al.*, 2007).

1.3. Oceanographic Currents.

The marine realm allows many marine species to be widely distributed, through the dispersal of larvae, migration and aggregation (Roff & Zacharias, 2011). As referred previously, genetic studies can provide valuable information about connectivity patterns between populations; ocean currents could help to spatially predict how the connectivity occurs (Roff & Zacharias, 2011). Therefore, the general pattern of oceanographic surface circulation in our sampling distribution area is described below.

1.3.1. Northern Atlantic Ocean Surface Currents

North Atlantic Current (NAC) reaches the meridian zone of the North Atlantic (Figure 2) divided in two main branches, one as a Northeast Drift Current called Eastern North Atlantic Central Water Subpolar mode (ENACWsp), and the other one as a Southeast Drift Current called Eastern North Atlantic Central Water subtropical (ENACWst) mode. Between the NAC modes there is a transition zone, where the Portugal Current System is formed (Pérez *et al.*, 2001).

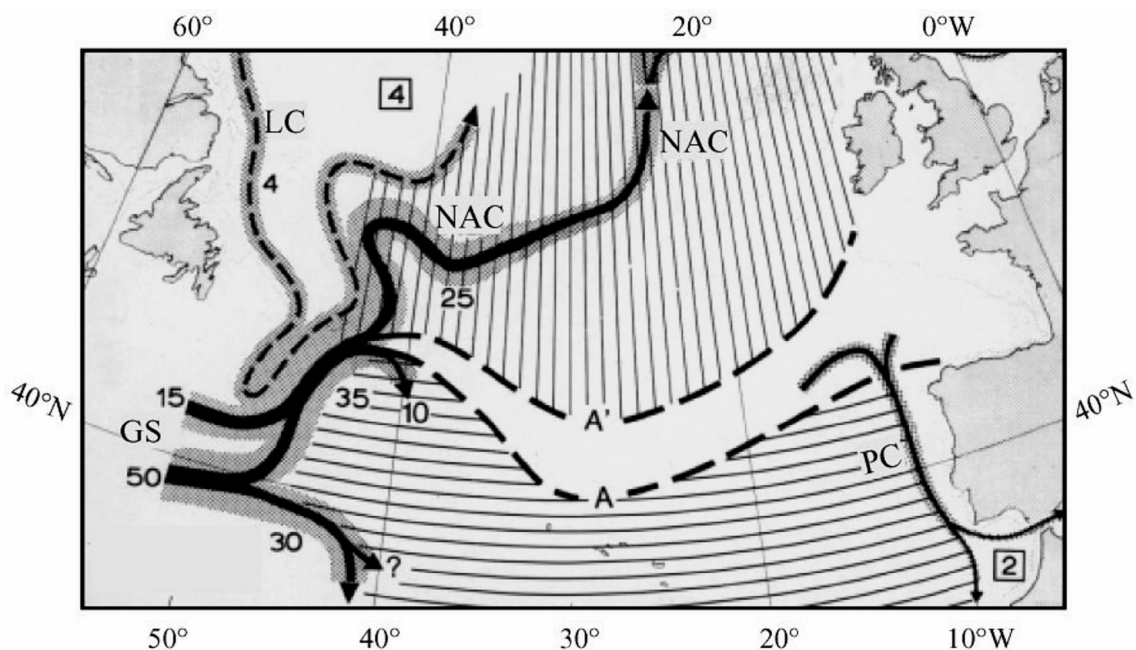


Figure 2- Surface circulation of the northern North Atlantic as derived from drift experiments (adapted from Krauss, 1986 in Pérez *et al.* (2001)). LC, Labrador Current; NAC, North Atlantic Current; GS, Gulf Stream; and PC, Portugal Current Meridionally-ruled area, flow associated with the NAC toward the north. Zonally-ruled area, flow associated with the northern limb of the Subtropical Front. Numbers are transports in Sverdrups ($1 \text{ Sv} = 10^6 \text{ m}^3 \text{ s}^{-1}$).

This system is composed by a slow, offshore, basin scale equator-ward flow (Portugal Current, PC) and a fast, Iberian slope scale, seasonally reversing coastal flow, the Portugal Coastal Current. During Autumn/Winter, predominance of strong south-southwest winds, favours coastal convergence of surface circulation northwards, transporting warmer subtropical waters (ENACWst) over the upper slope and shelf break (aprox. 200 m deep) and preventing the off-shelf export of coastal water masses (Álvarez-Salgado *et al.*, 2003). This surface poleward current is called the Portugal Coastal Counter-current (PCCC) and it is driven by the large scale atmospheric pressure distribution in the North Atlantic (Coelho *et al.*, 2002).

In late spring/summer, the increase in the pressure gradient between the Azores and Portugal induces the formation of north/northwestern winds, shutting down the

PCCC, and originating instead, a coastal upwelling and a southward surface current in the vicinity of the shelf break, the Portugal Coastal current (PCC). The PCC transports recently up-welled cold and nutrient rich ENACWsp in the north ($>45^{\circ}$ N), and warmer and nutrient poor ENACWst ($<40^{\circ}$ N) in the south.

However, recent studies ((Relvas *et al.*, 2007; Nolasco *et al.*, 2013) point out evidences that PCCC (in these studies designated as the Iberian poleward current, IPC) is active during summer, suggesting interannual variability of the oceanographic regimes in the Iberian Coast.

Also on the Portuguese coast an undercurrent mixed with ENACWst and mediterranean outflow waters, extends vertically from 1500m to the bottom of surface mixed layer (Ambar (1986) in Coelho *et al.*, 2002) and moves poleward permanently (Coelho *et al.*, 2002). This subsurface occasionally reaches surface along the west and southwest coast, particularly in winter (Álvarez-Salgado *et al.*, 2003).

At the southeastern coast of Iberia (Figure 3), the Portuguese-Canary eastern boundary currents moves eastward into the Gulf of Cadiz, performing a quasi-permanent cyclonic eddy near the Cape of São Vicente, and moving further east towards the Strait of Gibraltar, feeding the Atlantic inflow into the Mediterranean Sea (Criado-Aldeanueva *et al.*, 2006; García-Lafuente *et al.*, 2006), and part, moves southward re-joining the Canary current.

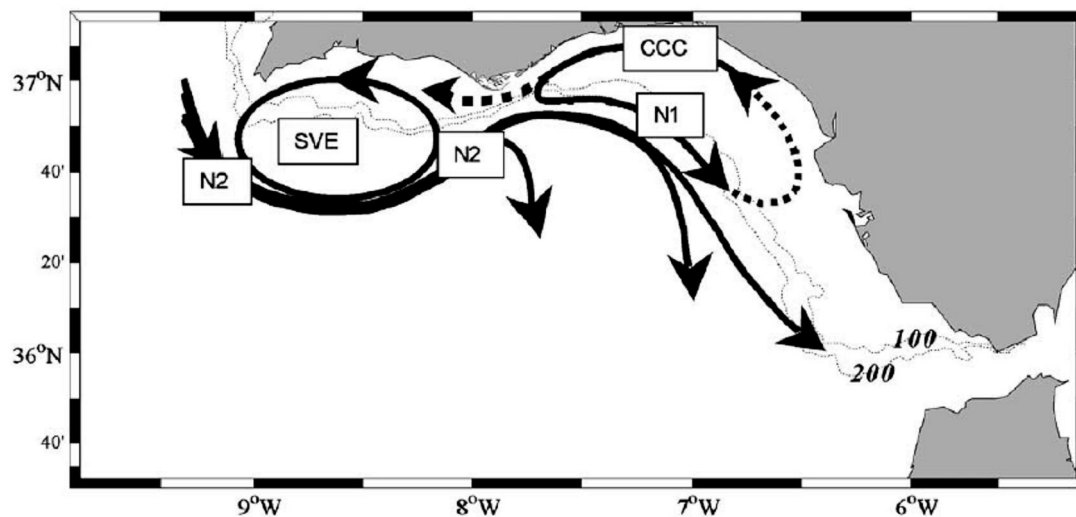


Figure 3 6 Scheme of the surface circulations in the Southern Iberian Coast and the Portuguese-Canary eastern boundary current that veers eastward into the Gulf of Cadiz, and eventually feeding the Mediterranean Sea. N2 is a branch of the larger-scale Portuguese-Canary eastern boundary current; SVE is the Cyclonic eddy off Cape San Vicente; N1 is cyclonic circulation bounded by a shelf break front; CCC is a warmer coastal counter current.

Source: Adapted from García-Lafuente *et al.* (2006).

On northeastern Africa, the Portugal current (PC) and the Azores current, will feed the Canary Current (CC). This equator-ward, broad, yearlong surface flow, is influenced by summer upwelling events, in response to the trade winds. Further south, in the vicinity of the Cape Verde Islands, this current is divided curving toward the west helping to create the North Equatorial Current (Batteen *et al.*, 2000; Barton, 2001).

1.3.2. Mediterranean Sea-Overall Functioning

This semi-enclosed sea is strongly affected by evaporation, precipitation, and river runoff (Millot & Taupier-letage, 2005). The Mediterranean Sea evaporation is the core engine that promotes the entrance of Atlantic Waters (AW) through the Strait of Gibraltar, in order to balance the difference in sea water levels between Northeastern Atlantic Ocean and Mediterranean Sea.

When AW passes through Gibraltar strait is modified increasing density due to evaporation, and suffering temperature changes due to climate seasonality. In winter, Northwestern and Northeastern Mediterranean basins, suffer marked cooling and evaporation, induced by cold dry air masses and strong northerly winds (Millot & Taupier-Letage, 2005). AW sink and mix with denser waters underneath creating the Levantine intermediate waters (LIW) and the Deep Mediterranean Waters (MWs).

Considering the volume of AW entering through Gibraltar Strait, it is estimated that 90% of this water mass forms the LIW and MWs (10% evaporates), showing an average residence time of 50 to 100 years (Millot & Taupier-Letage, 2005). Finally, these colder, saltier and denser MWs outflow and diverge deeper in the northern Atlantic Ocean, where it can be recognised at 1000-1200 meters.

1.3.2.1. Western Mediterranean Sea Surface Currents

Western Mediterranean Sea (WMS) surface circulation is mainly driven by the inflow of AW (*i.e.* Atlantic jet, AJ) through the Strait of Gibraltar, wind patterns (André, G., P. Garreau, V. Vernier, 2005) orographic features and evaporation, producing different circulation regimes, seasonally and inter-annually. Therefore, we will consider in detail only the most prevailing circulation systems during summer and early autumn, covering the main reproduction season and larvae dispersal phase of *Holothuria mammata*.

The influx of AW moves firstly northeastwards due to the orientation of the strait, and usually starts describing a year around persistent clockwise gyre in the west of the Alborán Sea, designated by Western Anticyclonic Gyre (WAG) and a second, less intense and recurrent clockwise gyre at east of Alborán Sea, called Eastern Anticyclone Gyre (EAG). Both Gyres are separated by the Alborán Ridge, and confined between the strait of Gibraltar at western limit and the Almeria-Orán front forming the eastern limit of the Alborán circulation system (Renault *et al.*, 2012).

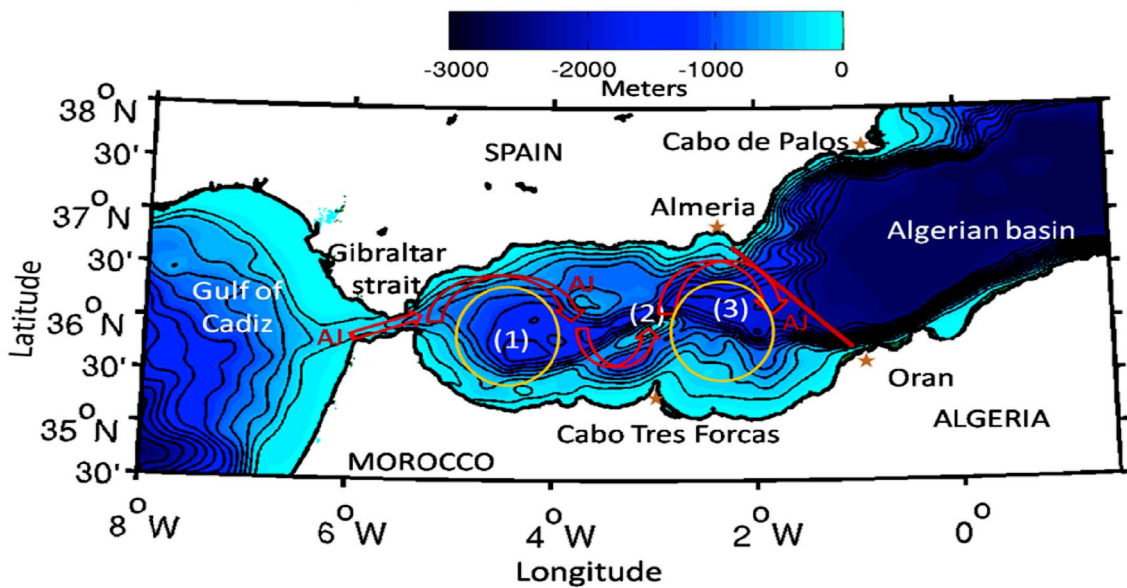


Figure 4- The red line represents Almeria-Orán front. The red arrows represent the boundary of the Atlantic Jet. And the orange circles display the Western Anticyclonic Gyre (WAG) and the Eastern Anticyclonic Gyre (EAG) positions, the numbers 1, 2, 3 in parentheses refer to the Western Alborán basin, the Alborán ridge, and the Eastern Alborán basin, respectively. Adapted from Renault *et al.* (2012).

The WAG and EAG circulation regime prevails in summers months (Figure 4), when AW kinetic energy (KE) is stronger, the single anticyclonic gyre (WAG) regime is more recurrent in winter months, when KE decreases (Millot & Taupier-Letage, 2005; Renault *et al.*, 2012). The Almeria-Orán frontal zone separates this relatively fresh surface water mass with Atlantic origin, from the denser and modified Mediterranean water mass of the Algerian basin (Renault *et al.*, 2012).

After restructuring itself due to the Coriolis effect, (Millot & Taupier-Letage, 2005), the AJ forms two main branches, the first one which is predominant when the EAG is well defined (*i.e.* in late Summer-early Autumn), flows into the Algerian sub-basin forming the Algerian current (AC), and the second branch (Figure 5) that inflows to the Northwestern sub-basin, through the Ibiza and Mallorca Channel, forming the Balearic Current (BC). The AC flows from Alborán Sea to the east along continental

slope of northern coast of Africa (Sayol *et al.*, 2013) and the BC flows along the northwest coast of the Balearic Islands (Heslop *et al.*, 2012).

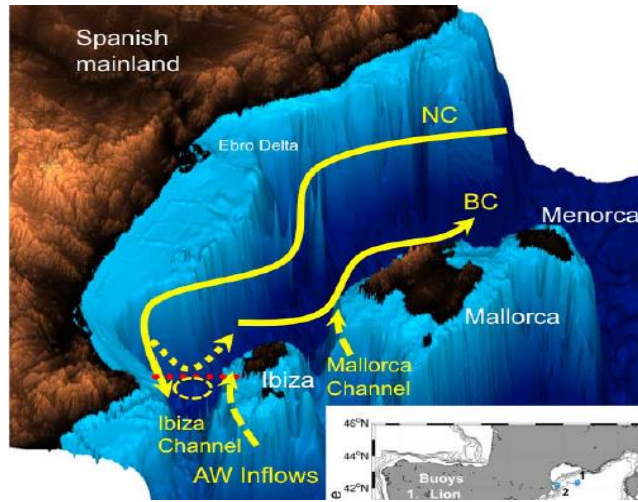


Figure 5- The Balearic Sea with main currents Northern Current (NC), Balearic Current (BC) and AW inflows through the Ibiza and Mallorca Channels. A -blocking eddy (yellow circle dashed line) and the re-circulation of the NC above the eddy (yellow dashed line) are also indicated. Adapted from: Heslop *et al.* (2012).

The intensity of the EAG and Northern Current (NC) modulates the circulation to the eastern (to form the AC) as well as the inflow of AW to the Northwestern sub-basin (to form the BC), being only in summer when the NC decreases, that southern waters progressively invade the Balearic sub-basin (Pinot *et al.*, 2002; Sayol *et al.*, 2013).

The AC will generate wide cyclonic open sea eddies, some of them reaching the Balearic Islands (Millot & Taupier-Letage, 2005), but will continue to transport AW along slope, part through the western coast of Corsica forming the Corsica Vein, and the other part through the Channel of Sardinia to form the Tyrrhenian vein and Eastern Mediterranean vein (Figure 6). Both (Corsica, Tyrrhenian Vein) water masses will join at the Ligurian sea, reorganizing itself again as the Western Basin Gyre. This gyre will continue north-westerly along slope, forming the Northern Current near the Gulf of Lions. This modified cold saline water mass, will proceed southward, closing its cycle when exiting to the North Western sub-basin through the Ibiza channel, or/and being retroflected cyclonically to form, along with the inflow of AW, the Balearic Current (Sayol *et al.*, 2013).

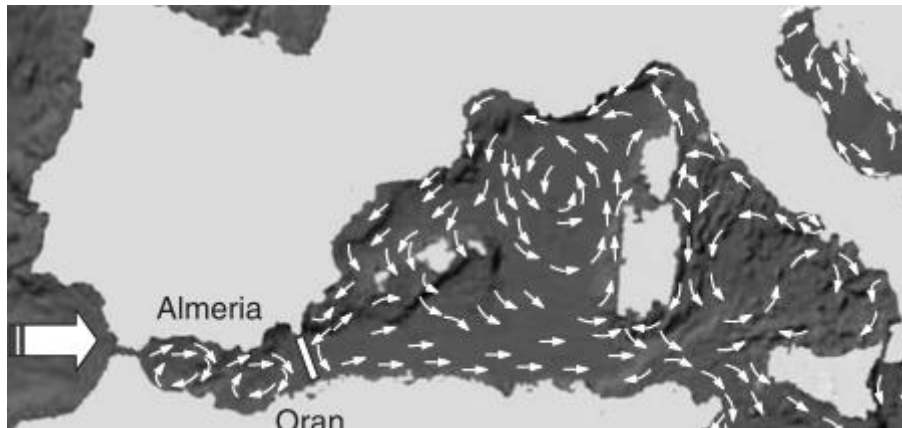


Figure 6 - Schematic representation of the main currents characterizing water circulation in the Western Mediterranean. Adapted from: Patarnello *et al.* (2007).

1.3.2.2. Eastern Mediterranean Surface Currents

After entering the Channel of Sicily, AW circulates southward, branching into Tunisia coast and shelf veins, and a third inter-annual meandering vein that flows northerly into the Ionian Sea, forming mesoscales eddies (Figure 7) (Hamad *et al.*, 2005; Millot & Taupier-Letage, 2005). At Libia, both parts of Tunisian vein merge hence becoming a Libyan vein, moving along slope in eastward direction. Instability dominates the Libyan basin, promoting eddies that moves away from the Coast to southern Ionian (Hamad *et al.*, 2005). The δ Libyo-Egyptian Current δ develop anticyclonic eddies, that moves and accumulate in the Western Levantine feature (\hat{U}_{LW}). Instability of the AW circulation in the Middle East slope, also promotes small scale eddies that accumulate and decay in the Eastern Levantine feature (\hat{U}_{LE}). From the Turkish slope until the Rhode Island, the Asia Minor current generates meanders, some of them evolving to small eddies (Millot & Taupier-Letage, 2005). The Eastern basin gyre at the Rhode Island enters the Aegean Sea and divides into a Northwestern and a Southwestern veins. The Northwestern vein flows along the Turkish slope most of the year, except in summer, where a sub-vein flows southward due to Melten winds and join the black sea outflow. The Southwestern vein moves along southern slope of Crete until Ierapetra, but then it flows toward \hat{U}_{LW} and again around the eastern Levantine. Therefore most of AW exits via the Aegean west of Crete. AW continues northwards trough Ionian Sea, where it re-constitutes the eastern gyre before bifurcating into a northern vein that penetrates into the Adriatic and mixes with river run-off and eventually joins the westward vein that bypassed the Adriatic. Both ones continue along

the southern tip of the Italian peninsula and Sicily closing the Eastern Basin gyre circuit (Hamad *et al.*, 2005).

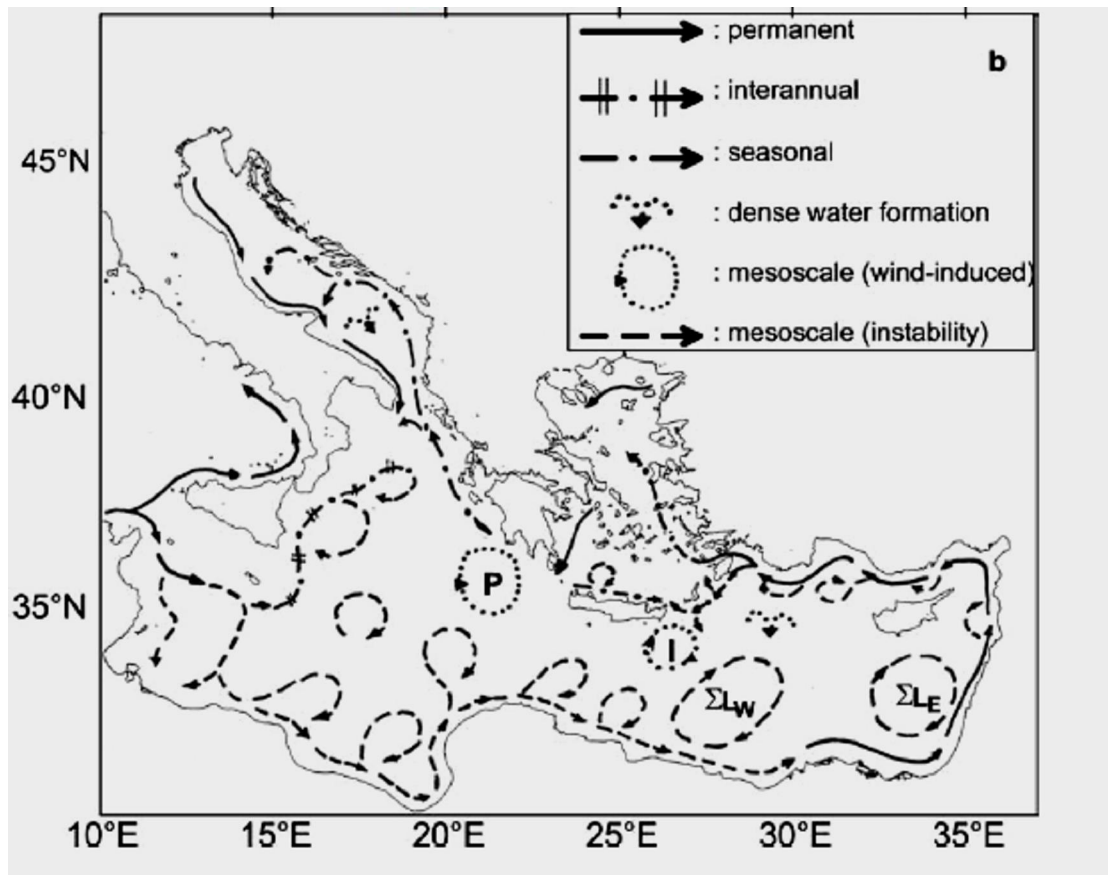


Figure 7 - Surface circulation scheme in the eastern Mediterranean basin, the Western Levantine feature ($\hat{\Sigma}LW$), and the Eastern Levantine feature ($\hat{\Sigma}LE$). Adapted from: Hamad *et al.* (2005)

1.4. Study Case: Sea Cucumbers.

1.4.1. Ecological Role

Sea cucumbers comprise more than 1400 species worldwide belonging to 160 genus (Navarro, 2012). Ecologically, holothurians are important deposit and filter feeders having significant roles in recycling nutrients, stimulating microalgae growth, and mixing the upper sediment layers (MacTavish *et al.*, 2012).

In benthic communities, they act as recyclers of nutrients and are agents of bioturbation (Uthicke, 1999), allowing the oxygenation and preventing the stratification and hardening of sediments (Bruckner *et al.*, 2003). Moreover, they actively feed on benthic microalgae, fungi and bacteria, avoiding excessive development of

microorganisms, increasing the aerobic layer of sediments and avoiding eutrophication (Uthicke, 1999; Hamel & Mercier, 2008).

Some sea cucumbers consume large quantities of suspension or sediment organic matter, converging organic detritus and releasing nitrogenous compounds which can be used by algae (Uthicke & Klumpp, 1998; Uthicke, 2001) and seagrasses (Wolkenhauer *et al.*, 2010), increasing their productivity, thus producing more available food for herbivores (Uthicke, 2001). Removal of sea cucumbers in some habitats may therefore reduce primary production for the whole food chain and negatively affect sediment infauna by reducing the aerobic layer of sediments (Uthicke, 1999; Purcell, 2013). Some sea cucumbers showing suspensivorous feeding are also helping to regulate water quality, carbonate content and pH of the water (Massin, 1982). Holothurians are also important preys for fish, seastars and crustaceans (Francour, 1997).

1.4.2. Food Potential and Sea Cucumber Fisheries

The decline of several traditional fin-fisheries (Hutchings & Baum, 2005; Pauly *et al.*, 2005; Eldridge *et al.*, 2009), brings our concern to one heavily exploited invertebrate: sea cucumbers. They are also known as *õbeche-de-mer*, *õ õtrepang*, *õ õhaisomõ* and are mainly an Asiatic feeding custom, (Chen, 2003). They have been a popular luxury food item in the Asian dried seafood markets for centuries (Purcell, 2013), but also considered as medicine (Chen, 2003) and aphrodisiac (Ramón *et al.*, 2010a). Holothurian nutritional profiles have demonstrated the presence of high protein content, antioxidant and bioactive compounds (Bordbar *et al.*, 2011; Roggatz, 2012).

Records from their utilization can be traced back to 1368õ1644 BC in China (Chen, 2003) but during this last millennium the Indo-Pacific regions have started to harvest and trade sea cucumbers driven primarily by the Chinese demand (Anderson *et al.*, 2011b). During the last 6 decades sea cucumber fisheries have grown exponentially with an increase in global captures of 2300 tonnes of wet weight in 1950 to 30 500 tonnes of wet weight in 2006 (Conand & Bryne, 1993; Anderson *et al.*, 2011b) and 100 000 tonnes in 2010 (FAO, 2010). In 2000, about 6 000 tonnes of processed (*i.e.* mostly dried) animals were exported to Asian markets, having a value over 130 million US dollars (FAO, 2010).

Nowadays, at least 3 million fishers from 70 countries worldwide capture sea cucumber (Purcell, 2013), in which most of landings are exported to Hong Kong and then re-exported mainly to China (Anderson *et al.*, 2011b), with some target species like *Apostichopus japonicus* reaching almost US\$ 3000/kg (Purcell *et al.*, 2012) (Figure 8).

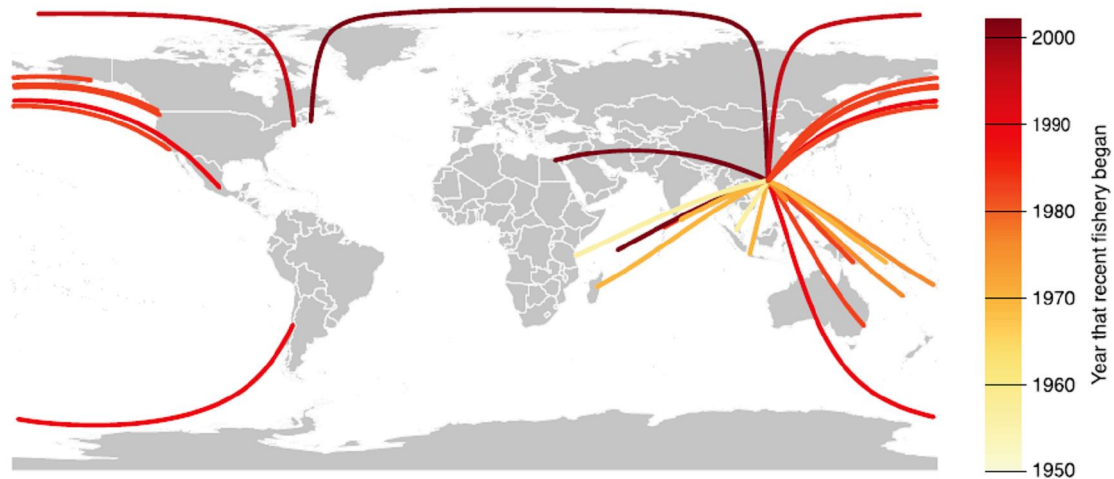


Figure 8 - Map of global sea cucumber catch as exported to Hong Kong. Lines indicate great circle arc between the cities with the largest population in each country or region and Hong Kong. Colour reflects the starting year of the recent fishery. Source : Adapted from Anderson *et al.* (2011).

Although 66 species of sea cucumbers represent global captures of about 100 000 tonnes annually (FAO, 2010) there is a lack of biological information for some of these target species (Ramón *et al.*, 2010). In general, sea cucumbers have some biological traits that makes them quite vulnerable to fisheries such as slow growth rate, late age at maturity, high larval mortality, low rate of recruitment, slow rate of movement and easy access by fisherman (Uthicke *et al.*, 2004; FAO, 2010; Anderson *et al.*, 2011b; Purcell *et al.*, 2013). Moreover, due to their synchronized gonochoric broadcast spawning behaviour, their fertilization success is population density-dependent, meaning that if densities are reduced by 50 % , it can result in a reduction of more than 50% in recruitment (Uthicke & Conand, 2005), a process called *Allee effect* (Allee, 1938).

Purcell *et al.* (2013) carried out a global analysis assessment, finding that 58% of sea cucumber fisheries were overexploited (contrasting with the 30% overexploitation of global stocks of fish (FAO, 2012)), 14% fully exploited, and 27 % non-fully exploited (Figure 9)

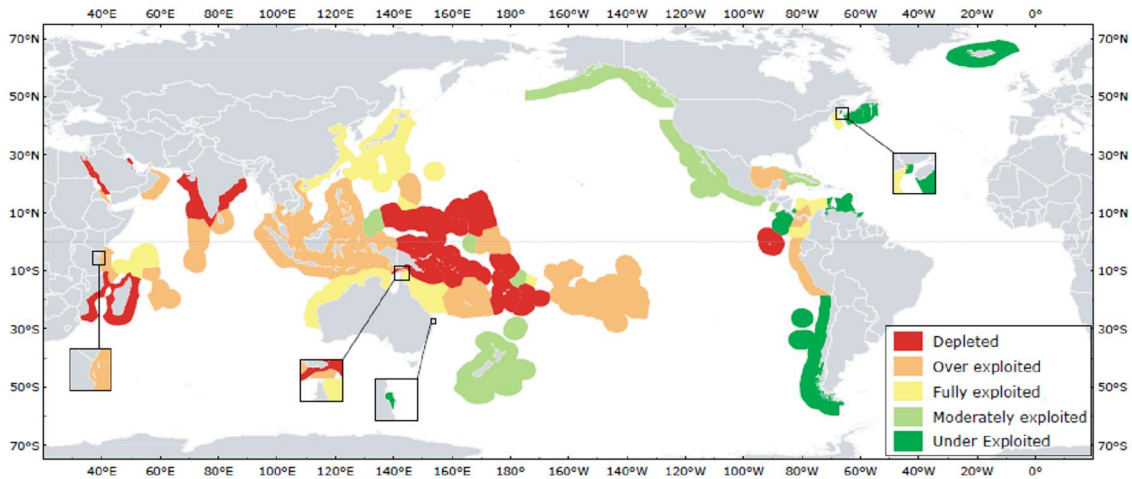


Figure 9 - Current status of sea cucumber fisheries in global fisheries (n° of fisheries= 69). Source: adapted from Purcell *et al.* (2013).

Considering the ever increased demand by Asian consumers and that most Indo-Pacific fisheries of sea cucumber are overexploited, depleted or in a moratorium state, countries already started to explore new target sea cucumber species, with increasing popularity in Asian market (Sicuro & Levine, 2011). Fisheries of sea cucumber are being developed in Northeastern Atlantic Ocean and Mediterranean Sea (Sicuro & Levine, 2011; González-Wangüemert *et al.*, 2013b, 2015) being their main target species *H. sanctori*, *H. mammata*, *H. tubulosa*, *H. polii*, *H. arenicola* and *Paratichopus regalis* (Abdel Razek *et al.*, 2007; Aydin, 2008).

In Turkey, harvest and exportation to Asian market rapidly increased from 20 tonnes of sea cucumber in 2002 (Aydin, 2008) to 555 tons in 2012 (González-Wangüemert *et al.*, 2014a), provoking the reduction of genetic diversity, size and weight of individuals from the main fishery areas (González-Wangüemert *et al.*, 2014; 2015). Italy has initiated small-scale harvesting and processing sea cucumbers for exportation and local consumption mainly in southern regions (Sicuro & Levine, 2011; Sicuro *et al.*, 2012). Spain has been traditionally harvesting *P. regalis* which is considered a delicacy, and the most valued seafood in the Catalan market, reaching up to €130/kg (Sicuro & Levine, 2011; González-Wangüemert *et al.*, 2014b; Maggi & González-Wangüemert, 2015). In Spain, more than 10 companies are exporting sea cucumbers (*H. tubulosa*, *H. forskali*, *H. mammata*) to China (<http://www.alibaba.com/countrysearch/ES/sea-cucumber-supplier.html>), some of them with 1-2 millions \$ US of total revenue. In Portugal, several companies (e.g. Omegauasia Lda) are selling sea cucumbers, mainly *Holothuria arguinensis*, *H. sanctori*, *H. forskali* and *H. mammata*, offering supply ability among 2.000-50.000 Kg/month and prices

oscillating among 70-350 euro/kg (<http://www.alibaba.com/countrysearch/PT/sea-cucumber.html>) depending on quality of product.

All these data show that sea cucumber fisheries in the Mediterranean Sea and Northeastern Atlantic Ocean are starting to become an active industry. Given that most sea cucumber fisheries worldwide have not had an effective management due to the lack of biological, genetic and ecological data (among another reasons), it is mandatory to initiate studies focused on these new target species of sea cucumbers from the Mediterranean Sea and Northeastern Atlantic Ocean in order to assess their growth, mortality rates, longevity, reproductive biology, larval duration, genetic diversity, connectivity and genetic structure. All this information will allow a better understanding of the sea cucumber population dynamics, fishery effects and the delimitation of their stocks as management units.

1.4.3. Pharmacological Potential

Although the Chinese people have been using sea cucumber for medicinal purposes since more than 3000 years (Chen, 2003), only recently it has been demonstrated the antimicrobial, antioxidant, and anticancer potential of sea cucumbers (Sicuro & Levine, 2011; Roggatz, 2012). New compounds are being discovered, such as anti-malaria and anti-HIV therapy drugs (Sicuro & Levine, 2011) and also new biochemical compounds for fighting cancer with less toxicity than traditional ones (Attoub *et al.*, 2013).

1.4.4. Integrated Multi-Trophic Aquaculture Potential

Aquaculture is one of the fastest-growing food producing sectors in the World, providing almost half of all fish for human consumption, and it is projected to rise to 62 % in 2030 (FAO, 2014), a near future in which the human population is expected to reach around 8.3 billion people (United Nations, 2013).

With increasing fishing pressure on wild stocks worldwide, responsible and regulated aquaculture development seems a sustainable way of supplying part of the demand from worldwide markets, releasing the pressure from wild populations and supporting efforts to preserve natural populations. However, standard aquaculture systems produce a large amount of particulate organic matter derived from unconsumed

feed, faeces and inorganic nutrient excretions (Troell *et al.*, 2003; Slater & Carton, 2007; Yokoyama, 2013). This could result in excessive release of dissolved nutrients into the water column, promoting harmful algal blooms and poisoning the farmed fish and surroundings (Neori *et al.*, 2004; Imai *et al.*, 2006). Moreover the increased bio-deposition below the fish cages is known to change the chemistry of the bottom (Richardson, 2008; Yokoyama, 2013), shifting in most cases the composition of benthic communities from oxygen sensitive species to opportunistic species (Troell *et al.*, 2003).

Integrated Multi-Trophic Aquaculture (IMTA) is a promising alternative that reassures greater environmental stewardship while increasing economic benefits for farmers and communities. The underlying principle is based on an ecosystem approach and involves growing several commercial species, with different trophic levels but with direct trophic linkage in the same system (Hughes & Kelly, 2006; Barrington *et al.*, 2009). On this way uneaten feed, wastes, nutrients and by-products of fed species (*e.g.* finfish, shrimp) could be recaptured and converted into fertilizer, feed and energy for the growth of organic extractive species as filter feeders (*e.g.* mussels) and deposit feeders (*e.g.* sea urchins, sea cucumbers) and inorganic extractive species (*e.g.* sea weeds). IMTA has the advantage of providing diverse and safe food products (Neori *et al.*, 2004), extra income to farmers (Troell *et al.*, 2003; Yokoyama, 2013) and mitigation of the environmental impacts caused by aquaculture facilities, increasing the carrying capacity of farming areas and consequentially improving the overall health of the site with minimum potential risks to wild and cultured species (Rawson *et al.*, 2002; Barrington *et al.*, 2009). Due to their ecological characteristics (*i.e.* filter and/or deposit feeders), high global demand and alarming levels of overfishing, sea cucumbers are a suitable candidate for co-culture in the recently developing IMTA systems (Slater & Carton, 2007; Nelson *et al.*, 2012; Yokoyama, 2013; Domínguez-Godino *et al.*, 2015), mainly because they can fill one of the main ecological niche of IMTA by filtering the larger organic matter particles (Nelson *et al.*, 2012).

1.5. Biological Model: *Holothuria mammata* (Grube, 1840)

Sea cucumbers are invertebrates from the phylum Echinodermata, having a limbless soft bodied, with a cucumber form. The genus *Holothuria* belongs to family

Holothuriidae and is one of the most diverse genus in the Holothuroidea class (Borrero-Pérez *et al.*, 2010).



Figure 10 - Adult sea cucumber *Holothuria mammata*.

Holothuria mammata is a temperate species occurring throughout the Mediterranean Sea and Northeast Atlantic Ocean, including the continental Atlantic coast of Portugal and the Macaronesian Islands of the Azores, Madeira and Canary Islands (Borrero-Pérez *et al.*, 2009) (Figure 11).

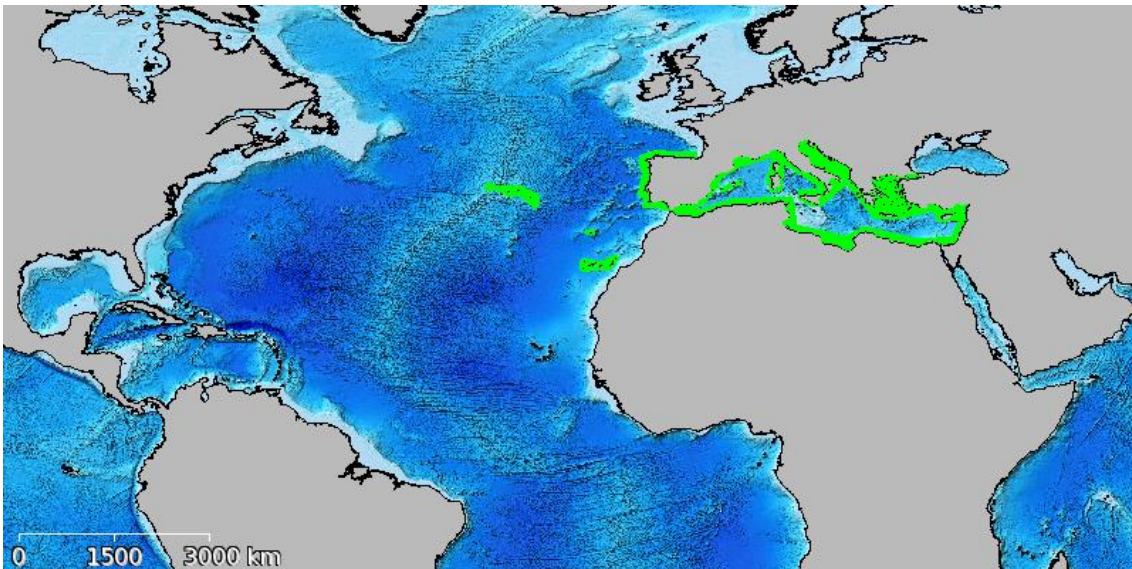


Figure 11 - Geographical distribution of *Holothuria mammata*. Source: adapted from CUMFISH Webpage.

H. mammata coloration has scarce variation, with uniform dark purplish brown in dorsal and ventral side and brighter tube feet and papillae (Figure 10). The ventral

side is densely covered by pedicles which are organized in 5 rows. The species has small Cuvierian tubules but few in number and never expelled (*Borrero-Pérez et al.*, 2011; Navarro, 2012). Its length average is 23 cm, and may reach 35 cm in Canary islands (Navarro *et al.*, 2013), and ranged between 13 to 25cm in Ria Formosa (Siegenthaler, 2013).

They can be normally found within rocks crevices showing mainly nocturnal activity (Navarro, 2012; Navarro *et al.*, 2013). Despite its rocky habitat preference, *H. mammata* was also found on sandy/muddy bottoms with seagrass from Ria Formosa coastal lagoon (Portugal), although showing low densities (González-Wangüemert *et al.*, 2013a; Siegenthaler, 2013).

Scarce information is available about the reproduction or larval duration on this species. Santos (2013) established that the maximum sexual maturation state (Gonadosomatic Index, GSI) of *H. mammata* from Peniche (W Portugal) is reached only in April, suggesting a single annual spawning season in this region.

Recent breeding and rearing trials, showed that they have a broadcast spawning behaviour in mid-summer with a planktotrophic larval cycle duration of 19 days until the pentactula settlement stage (Dominguez-Godino, personal communication).

2. Objectives

The main purpose of this study is to characterize genetically populations of *Holothuria mammata* from 8 localities, four of them belonging geographically to Northeastern Atlantic, and the other ones to Mediterranean Sea. Its genetic diversity and structure will be analysed considering several spatial scales.

Moreover, morphometric data (weight vs. length) will be studied through their frequency distributions, in order to implement baseline data for future morphological comparisons between stocks with different fishery pressure. This assessment will be fundamental to initiate proper management policies in order to conserve this novel target species. Therefore, this work is focused on these particular aims:

1. To evaluate the genetic diversity patterns of *H. mammata* through its geographical distribution.
2. To assess the genetic structure of this target species at different spatial scales considering the role of current and historical barriers to gene flow in several potential genetic breaks: Atlantic and Mediterranean basins, Western and Eastern Mediterranean Sea, geographical isolation of the oceanic islands (Canary archipelago) and isolation among coastal vs. islands samples.
3. To establish the potential stocks present in our study area considering the genetic and morphological data.
4. To estimate the effective size of *H. mammata* populations.
5. To evaluate the status of the target species by analysing the distribution of size and weight classes of their populations.
6. To discuss potential impacts of fisheries on *H. mammata* under genetic and morphological perspectives.
7. To suggest first recommendations towards sustainable management of the *H. mammata* fishery in Mediterranean Sea and Northeastern Atlantic Ocean.

3. Methodology

3.1. Sampling

The samples were caught under the CUMFISH project (PTDC/MAR/119363/2010) in which all individuals were collected by scuba diving and free diving between 2011 and 2013. Thirty-five individuals were sampled from each locality (Figure 12), except for Canary Islands where only 20 individuals were caught because this location is considered the south boundary of *H. mammata* distribution, being its density there lower than in other locations. *Holoturia mammata* individuals were identified on the basis of external characters and habitat, sacrificed by freezing and preserved in 100% ethanol until tissue dissection. Tissue samples of internal muscle bands or wall were removed from each specimen and preserved in 100% ethanol.

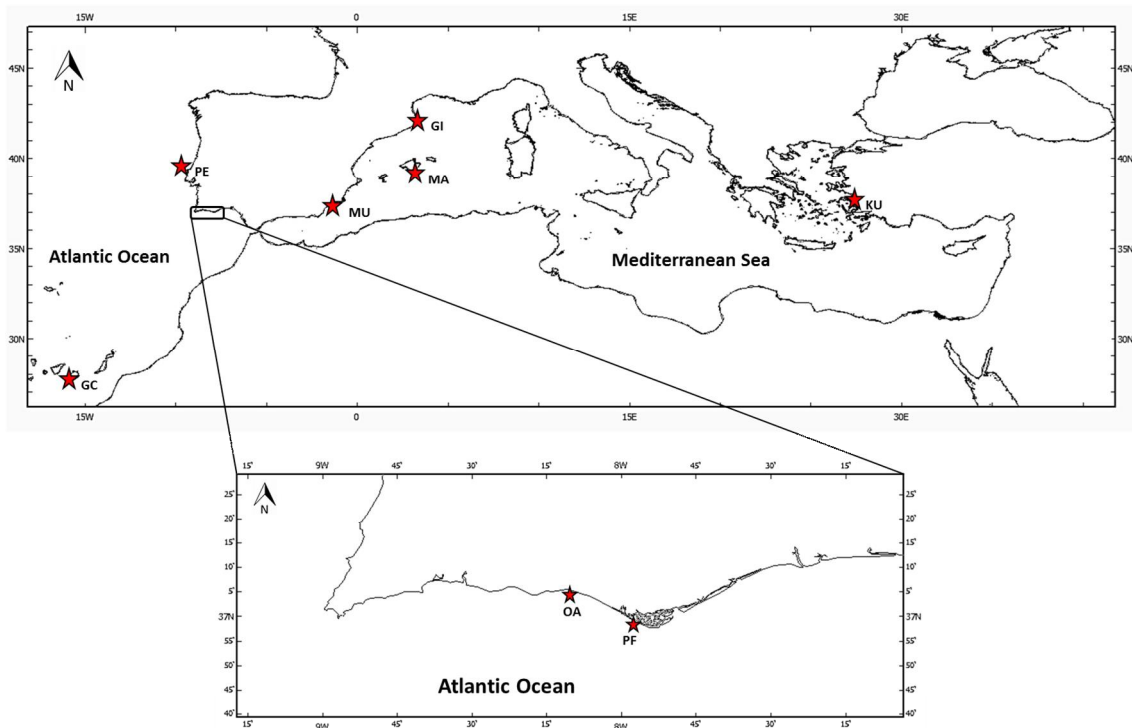


Figure 12 - Location of the sampling sites in the Atlantic Ocean and Mediterranean Sea. (GC: Gran Canaria; PE: Peniche; OA: Olhos de Água; PF: Faro; MU: Murcia; MA: Mallorca Island; GI: Girona; KU: Kusadasi).

3.2. DNA Extraction, PCR Amplification and Screening

Total genomic DNA was extracted from muscle tissues according to the procedure of Sambrook *et al.* (1989), with minor modifications.

The development of the *Holothuria mammata* specific primers for microsatellites, was performed by Ecogenics[®] from 15 samples belonging to different locations. Size selected fragments from genomic DNA were enriched for SSR content by using magnetic streptavidin beads and biotin-labeled CT and GT repeat oligonucleotides. The SSR enriched library was analyzed on a Roche 454 platform using the GS FLX titanium reagents.

The total 7082 reads had an average length of 159 base pairs. Of these, 435 contained a microsatellite insert with a tetra- or a trinucleotide of at least 6 repeat units or a dinucleotide of at least 10 repeat units. Suitable primer design was possible in 138 reads, of which 52 were tested for polymorphism. Finally nine microsatellites were chosen according to feasibility and genetic diversity criteria (Table I).

Table I- Primers sequences, type of repeats, size, n° number of alleles found in 15 samples and annealing temperature for PCR amplification.

Locus	Primer sequences 5' 3'	Repeat type	Size bp°	N° of alleles	TA [°C]
Holmam_00008	F CGATGTTGAGCCATGACCAC R CGCTACTTGCGAGATGTCTAC	(GT) 15	64 -97	6	56
Holmam_00457	F GGGACCAAAAAGCAAAACAAAAC R GCCCAATCAAGTCGAAACCC	(AAAC)7	136 -144	3	56
Holmam_01787	F ATGCTTAGCTGGCTTGTGTG R CCTTCTTTGGCCATTAAGATGC	(TG)15	84 -114	9	56
Holmam_01843	F CGGTGCATGCCAGTTTG R GCCACGCCTATTACTTTCCC	(AC)12	76 -101	10	56
Holmam_02503	F AGAACAGAGAGTTGGTTGTAAGC R AGCAGTCACTCTAGAATCTCC	(TG)13	131-160	8	56
Holmam_03018	F CTGAGCAGCAACCTAATGCC R ACGCAACAAATTTACACGGAAG	(TG)13	69 -124	12	56
Holmam_03415	F CCATTGTTTAGGTCCTCGG R GATGGCCCACTGGTAGAGAG	(CA)13	180 -198	3	56
Holmam_05474	F ATACACACCCTCACCCACAC R AATGCCTCCTCCACGTAGC	(CA)13	105 -129	6	56
Holmam_06203	F TCTTTTAAGTGGCATTGTGTCC R TACCTTCTGCTCCTGACCTG	(AC) 12	106 -119	6	56

Samples from 248 individuals were screened for variation at each of ten microsatellite loci. Polymorphisms were tested by PCR, performed according to conditions shown in Table II.

Table II-PCR conditions, with reagents concentration, number of cycles and temperatures, respectively

Reagents	Stock concentration	Amount [l]	Final amount/ Concentration	PCR cycle	
ddH ₂ O	-	4.1	4.1 l		
Buffer solution*	10x	1	1x	95°C 15 min	30 Cycles
dNTPs	2 mM	1	200 M	95°C 30 s	
FW. Primer ^o	2 M	0,2	0,04 M	56°C 45 s	
RV. Primer	2 M	0,8	0,16 M	72°C 45 s	
M13 Primer ^{oo}	2 M	0,8	0,16 M		
Hotstar Taq	5 u/ l	0,1	0,5 u	95°C 30 s	8 Cycles
DNA	5 ng/ l	2	10 ng	53°C 45 s	
* Qiagen, buffer stock (10x) containing 15 mM MgCl ₂ ^o with 18 bp M13-tail (5'-TGTAACAACGACGGCCAGT-3') at 5'-end ^{oo} labelled with a fluorescent dye				72°C 45 s	
				72°C 30 min	
				10°C -	

Individuals were genotyped by assessing allele size on an ABI 3700 automated sequencer, using forward primers labelled with 6FAM (SIGMA), HEX (SIGMA) and NED (Applied Biosystems). Genotyping was performed through two multiplexes of five/four microsatellites each. Allele scoring was carried out using STRand v2.4.59.

3.3. Genetic Analysis

3.3.1. Genetic Diversity

As parameters of genetic diversity were considered: total and mean number of alleles, number of private alleles, allele frequencies, expected (H_e) and observed (H_o) heterozygosity. They were calculated using Genetix v.4.05.2 (Belkhir *et al.*, 1996-2004) Genetic linkage disequilibrium between locus pairs was estimated according to Weir and Cockerham (1979) and tested on contingency tables under the null hypothesis of independence ($P < 0.05$) (Genetix v. 4.05.2).

Deviations from Hardy-Weinberg (HWE) genotype proportions were characterized by F_{IS} and tested using exact test in the software Genepop version 4.2.1 (Rousset, 2008). The observed genotype frequencies which deviated significantly from HW proportions, were analysed through the program Micro-Checker v.2.2.3 (Van

Oosterhout *et al.*, 2004) in order to infer the most probable causes for those deviations. Since Micro-Checker' main assumption of HW equilibrium, overestimates null allele frequencies when there is natural inbreeding populations (*i.e.* when populations are not in HW proportions), estimations of null allele frequency for each population and locus was obtained through the Expectation Maximization (EM) algorithm of Dempster *et al.* (1977) implemented in FreeNA software (Chapuis & Estoup, 2007). Also, FreeNA was used for evaluating possible differences between estimations of global and pairwise F_{ST} values including (*i.e.* INA) and excluding null alleles (*i.e.* ENA), in order to decide the feasibility of adjusting genotypes in the data set.

To distinguish the natural inbreeding of the species from the presence of null alleles, INEST software v1.0 (Chybicki & Burczyk, 2009), was used, which uses an individual inbreeding approach model (IIM) to estimate simultaneously null allele frequencies at each locus and the average level of natural intra-population inbreeding. In order to use IIM model, 500 000 Markov Chain Monte Carlo (MCMC) iterations, 10 000 n-th updates (*i.e.* thinning), and a burn-in period of 50 000 were used. Then, it was performed a Bayesian procedure of model comparison. For this purpose INEST computes Deviance Information Criterion (DIC) for each model; according with Chybicki & Burczyk (2009), the lowest DIC is corresponding with the model best fitting to data.

3.3.2. Genetic Structure

Microsatellites markers are very variable, therefore a set of statistical approaches to assess the spatial genetic structure of *H. mammata* was used. First, the quantification of the differentiation among locations through F_{ST} (*i.e.* using the estimator of Weir & Cockerham (1984)) was performed in Arlequin version 3.5 (Excoffier & Lischer, 2010), and then we tested the allele-frequency heterogeneity using an exact test and the p-values to check the significance of genetic differentiation.

Tests of genic and genotypic differentiation (*i.e.* G-based) for all pairs of populations (Raymond & Rousset 1995a; Goudet *et al.* 1996) were performed on GENEPOP version 4.2.1 (Rousset, 2008). Cavalli-Sforza distances were also computed between pairwise samples. Levels of statistical significance were adjusted according to a sequential Bonferroni correction for multiple comparisons (Rice, 1989).

Genetic differences were also analysed using a principal component analysis (PCA) on the allelic frequencies by She *et al.* (1987) and implemented with the `ade4` package (Chessel *et al.*, 2004) in R software (R Development Core Team, 2013).

An analysis of molecular variance (AMOVA) was carried out to assess the component of genetic diversity attributable to (i) variance between regions/groups; (ii) variance among populations within regions/ groups; (iii) variance within populations. First, Mediterranean and Atlantic basins were considered as groups and later coastal and island groups. Also we considered edges and central geographical groups, and accordingly with F_{ST} results we grouped non-differentiated populations. Finally it was grouped populations according to biogeographic criteria. Arlequin v3.5 software was used to carry out these analyses.

Population structure was also inferred using STRUCTURE v.2.2 software by the method of Pritchard *et al.* (2000) from multilocus genotype data. Each K was replicated 10 times for 100 000 iterations after a burn-in period of 50 000, using sampling locations as prior information to assist clustering. Each simulation was performed using 1) the admixed ancestry model and 2) the correlated allele frequencies model. STRUCTURE results were analysed in the program STRUCTURE HARVESTER v0.6.94 (Earl & vonHoldt, 2012), to calculate the ad-hoc \hat{K} statistic suggested by Evanno *et al.* (2005), which takes into account the change in the log probability of the data, in order to choose the number of clusters that best fit the data.

3.3.3. Gene Flow

To assess potential connectivity patterns on this species, isolation by distance, the coefficient of correlation between genetic and geographic/oceanographic distances was calculated using the Mantel test (10,000 permutations; Mantel, 1967) implemented in Genetix software. The geographical distance (km) was computed as the coastline distance between continental sampled locations and as the straight geographical distance for island populations. The oceanographic distances (Km), was computed according to the main oceanographic surface currents.

To study gene flow among localities, migration rates based on maximum likelihood was obtained with the program MIGRATE v. 3.2.7 (Beerli & Felsenstein, 2001;). MIGRATE uses a Markov Chain Monte Carlo based (MCMC) approach to explore all possible gene genealogies, providing estimates of the population size and

migration rates accordingly with the data. The MCMC run consisted of 10 short chains (sampling 50 000 trees) and one long chain (sampling 500 000 trees) with a burn-in period of 10 000 trees.

For a better understanding of gene flow pattern between the sampled localities an assignment test was conducted using GeneClass2 software v.2.0 (Piry *et al.*, 2004) to determine the most likely geographic origin of each individual using a frequency based method (Paetkau *et al.*, 1995).

3.3.4. Bottlenecks

Previous studies using mtDNA markers have demonstrated changes in *H. mammata* population sizes (Borrero-Pérez *et al.*, 2011). For this reason, we tested the presence of recent population bottlenecks at our samples in the last $2N_e-4N_e$ generations (Cornuet & Luikart, 1996). Bottlenecks can be detected by the depletion of allele number and a transient heterozygosity excess. We used the Sign and Wilcoxon tests implemented in the program Bottleneck v.1.2.2 (Piry *et al.*, 1999). Computations were based on the infinite allele model (IAM) and the two phase model (TPM).

3.3.5. Selection

To assess whether any microsatellites loci are under selection, LOSITAN software v. 1.0.0 was used (<http://popgen.eu/soft/lositan/>) (Antao *et al.*, 2008). This program evaluates the relationship between F_{ST} and expected heterozygosity (H_E) to identify outlier loci. We performed 75 000 simulations with \neq neutral mean F_{ST} and \neq force mean F_{ST} to increase the reliability of the mean F_{ST} and the entire microsatellite dataset under the Infinite Allele Model (IAM) and Stepwise Mutation Model (SMM). We choose confidence intervals of 99% to carry out a more conservative test for selection.

3.4. Morphometry Analysis

The total length (TL) and eviscerated weight (EW) for each individual with an accuracy ± 0.1 cm and ± 0.1 g respectively were registered. To improve the accuracy of the measures, the weight was registered after the death, evisceration and total cleaning

of the sea cucumbers. 298 individuals of *H. mammata* from 8 sampling localities were used for morphometric analysis. Regarding the measurements from Kusadasi (Turkey), length was measured after evisceration by Dr. Mehmet Aydin (Ordu University, Turkey), instead of registering the length when specimens were alive. Therefore, to compare the data from this locality with the other ones, we obtained an estimated length (*i.e.* length measurement before death) from the length-weight relationship resulting from all the length-weight data of individuals collected from the other localities included in this study ($W = 5.62L \pm 31.01$).

The mean length and weight for each population/region is represented through bar graphics. Length-weight relationship (LWR) was established using the linear regression analysis where $EW = a + b(TL)$, in which a is the intercept of the regression, and b the slope (*i.e.* growth coefficient). The significance of the regression was assessed by F-statistic, and the b -value for each species was tested by the t-test to check if it was significantly different from the isometric growth (*i.e.* $b=3$). When b is different from 3 it reflects an allometric growth, which could be positive (*i.e.* when $b > 3$) or negative (*i.e.* when $b < 3$) (Sokal & Rohlf, 1981). Data from Kusadasi (Turkey) were disregarded for LWR analyses, since it would bias results towards a linear relationship between eviscerated weight data and estimated length.

One-way ANOVA was used to test differences in length and weight among localities and between Atlantic vs. Mediterranean regions, considering "locality / region" as factors. Pairwise comparisons followed by *Bonferroni* adjustment were used to determine which group differences were statistically significant. All the analysis were performed in R statistic software (R Development Core Team, 2013).

4. Results

4.1. Genetic Diversity and Linkage Disequilibrium

All nine microsatellites revealed high polymorphism (Table III), with a total of 199 different alleles. Number of alleles per locus ranged from 13 at Holmam_00457 to 31 at Holmam_05474.

Table III: Primers sequences, type of repeats, size, n° number of alleles and annealing temperature for PCR amplification.

Locus		Primer sequences 5' - 3'	Repeat type	Size (bp)	Nº of alleles	TA [°C]
Holmam_00008	F	CGATGTTGAGCCATGACCAC	(GT) 15	61 -105	21	56
	R	CGCTACTTGCGAGATGTCTAC				
Holmam_00457	F	GGGACCAAAAAGCAAAACAAAAC	(AAAC)7	132 -176	13	56
	R	GCCCAATCAAGTCGAAACCC				
Holmam_01787	F	ATGCTTAGCTGGCTTGTGTG	(TG)15	87 -151	28	56
	R	CCTTCTTTGGCCATTAAGATGC				
Holmam_01843	F	CGGTGCATGCCAGTTTG	(AC)12	69 -119	21	56
	R	GCCACGCCTATTACTTTCC				
Holmam_02503	F	AGAACAGAGAGTTGGTTGTAAGC	(TG)13	130-190	19	56
	R	AGCAGTCACTCTAGAATCTCC				
Holmam_03018	F	CTGAGCAGCAACCTAATGCC	(TG)13	71 -155	29	56
	R	ACGCAACAAATTTACACGGAAG				
Holmam_03415	F	CCATTGTTAGGTCCTCGG	(CA)13	185 -217	16	56
	R	GATGGCCCACTGGTAGAGAG				
Holmam_05474	F	ATACACACCCTCACCCACAC	(CA)13	71 -167	31	56
	R	AATGTCCTCCTCCACGTAGC				
Holmam_06203	F	TCTTTAAGTGGCATTGTGTC	(AC) 12	104 -144	21	56
	R	TACCTTCTGCTCCTGACCTG				

Considering all populations (Table V), mean allelic richness for *Holothuria mammata* was 22.1 alleles, with Murcia (SE Spain) and Kusadasi (Turkey) showing the highest values (14.2 and 13.7, respectively) and Gran Canaria (SW Spain) presenting the lowest one (9.7), probably due to smaller sample size (Table V). We found 42 private alleles in total, with Kusadasi being the population with more private alleles (8) and Gran Canaria and Peniche, the localities with lower values (2 and 3 respectively) .

The observed and expected heterozygosity ranged from 0.501 (Olhos de Água, S Portugal) to 0.711 (Kusadasi, S Turkey) and from 0.758 (Faro, S Portugal) to 0.811 (Mallorca, E Spain), respectively. Inbreeding coefficient (F_{IS}) was positive and statistically significant in all sampling locations, indicating a possible deficit of heterozygotes (deviation from Hardy Weinberg equilibrium, HWE) (Table V).

Analysis of the most probable causes for deviations of HWE using Micro-Checker software, disregarded stuttering and large allele dropout, however several loci presented evidence for null alleles. Therefore, it was used freeNA software in order to compare F_{ST} values (Weir, 1996) from the original data set, and F_{ST} value obtained from

corrected data for null alleles, estimated by the ENA method (Chapuis & Estoup (2007)). There was no bias due to null alleles, as assessed when comparing ENA (*i.e.* excluding null alleles) global F_{ST} (0.0081) with INA (*i.e.* including null alleles) global F_{ST} (0.0089). Pairwise F_{ST} values (Table IV) with the INA and ENA showed similar trend, so we maintained the original data set for further analyses.

Table IV-Estimates of F_{ST} values (Weir, 1996) for each pair of *Holothuria mammata* populations considering (above diagonal) and not using (below diagonal) the ENA correction described in Chapuis and Estoup (2007).

Localities	PE	OA	FA	GC	MU	MA	GI	KU
Peniche (PE)	-----	0.003	0.006	0.003	0.006	0.006	0.015	0.022
Olhos de Água (OA)	0.002	-----	0.002	0.008	0.002	0.000	0.012	0.021
Faro (FA)	0.002	0.000	-----	0.000	0.002	0.006	0.016	0.021
Gran Canaria (GC)	0.000	0.002	0.000	-----	0.009	0.007	0.015	0.023
Murcia (MU)	0.007	0.000	0.001	0.008	-----	0.000	0.007	0.013
Mallorca (MA)	0.005	0.000	0.005	0.003	0.000	-----	0.006	0.013
Girona (GI)	0.018	0.012	0.019	0.017	0.007	0.005	-----	0.011
Kusadasi (KU)	0.026	0.019	0.024	0.026	0.013	0.011	0.010	-----

In order to better understand the significant positive F_{IS} values, INEST was used (Table V). The F_{IS} (obtained by INEST software) was positive in Gran Canaria and Mallorca, with values of 0.264 and 0.140 respectively and in Peniche with 0.110. The INEST model with the best fitting in Peniche and Mallorca, is considering the presence of null alleles and inbreeding. For Gran Canaria, the best model indicated that the inbreeding estimate calculated is due to populations' inbreeding and some genotyping errors. The model for the other localities, indicated null alleles as the only factor explaining the excess of homozygotes: the F_{IS} (INEST) was null on these populations (Table V).

Table V-Estimates of genetic diversity of the 8 sampling sites of *Holothuria mammata* from the Mediterranean Sea and Northeastern Atlantic Ocean.

Pop.	Sample size	Alleles	Allelic Richness	Private alleles	Ho	He	F_{IS} GENEPOP	HPDI< F_{IS} <HPDh INEST	M
PE	33	112	12.4	3	0,527	0,785	0.332*	0.000<0.110<0.273	n/f
OA	32	112	12.4	4	0,501	0,790	0.369*	0.000<0.000<0.000	n
FA	33	116	12.8	7	0,590	0,758	0.224*	0.000<0.000<0.000	n
GC	19	88	9.7	2	0,562	0,760	0.265*	0.191<0.264<0.348	f/b
MU	33	128	14.2	6	0,613	0,807	0.243*	0.000<0.000<0.000	n
MA	33	111	12.3	5	0,567	0,811	0.303*	0.023<0.140<0.257	n/f
GI	31	110	12.2	7	0,593	0,796	0.258*	0.000<0.000<0.000	n/b
KU	33	124	13,7	8	0,711	0,799	0.112*	0.000<0.000<0.000	n
Total	247	199	22.1	42	0,585	0,797	0.267*	0.009<0.044<0.092	n/f

Ho: observed heterozygosity; He: expected heterozygosity; F_{IS} : deviation from HardyWeinberg proportions.

* $P<0.001$. F_{IS} (INEST) is the mean unbiased inbreeding coefficient taking into account null alleles, and genotyping errors. M is the INEST model which best fit the data: n=null alleles, f=inbreeding, b=genotyping error.

HPDI / HPDh is the 95% highest posterior density interval.

Girona (NE Spain) and Faro (S Portugal) localities presented linkage disequilibrium between two loci, Holmam_01787 and Holmam_02503. Also, Holmam_01787 and Holmam_03018 loci presented linkage disequilibrium in Olhos de Água (S Portugal) and all Western Mediterranean populations (*i.e.* Murcia, Mallorca and Girona).

When a larger spatial scale was considered in order to analyse the genetic diversity pattern, interesting results were found: higher values on allelic richness (20.1 vs. 18.1), number of private alleles (35 vs. 19) and heterozygosity (0.62 vs 0.54) were registered in the Mediterranean region than the Atlantic one (Table VI).

Table VI- Estimates of genetic diversity of *Holothuria mammata* between the Mediterranean Sea and Northeastern Atlantic Ocean regions

Regions	Sample size	Alleles	Allelic Richness	Private alleles	Ho	He	F _{IS} GENEPOP	HPDI<F _{IS} <HPDh INEST	M
ATL	117	163	18.1	19	0.54	0.77	0.301***	0.000<0.000<0.000	n
MED	130	181	20.1	35	0.62	0.81	0.233***	0.000<0.000<0.000	n

H_O: observed heterozygosity; H_E: expected heterozygosity; F_{IS}: deviation from Hardy-Weinberg proportions.
 * P<0.001. F_{IS} (INEST) is the mean unbiased inbreeding coefficient taking into account null alleles, and genotyping errors. M is the INEST model which best fit the data: n=null alleles, f=inbreeding, b=genotyping error.
 HPDI / HPDh is the 95% highest posterior density interval.

Among the 10 polymorphic loci, Holmam_05474 appears as candidate for positive selection in both mutation models.

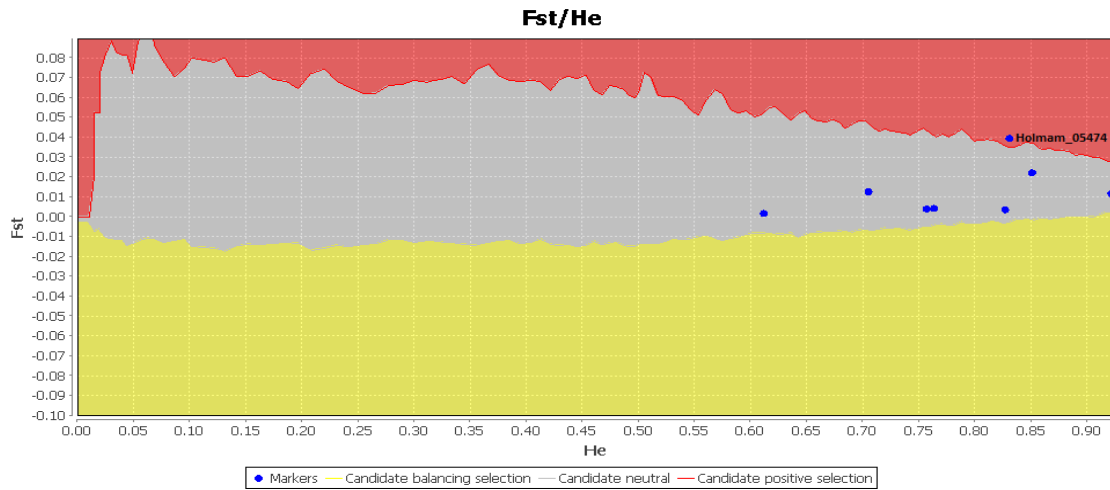


Figure 13 - Comparison of F_{ST} and He in polymorphic loci of *Holothuria mammata* to identify outliers and potential candidates for selection using LOSITAN software under the IAM. Graphical output shows the simulated confidence area for neutral loci (pale grey shading), positive selection (red area) and balancing selection (yellow bottom area). Loci outliers are tagged with labels. Locus Holmam_05474 is candidate for positive selection.

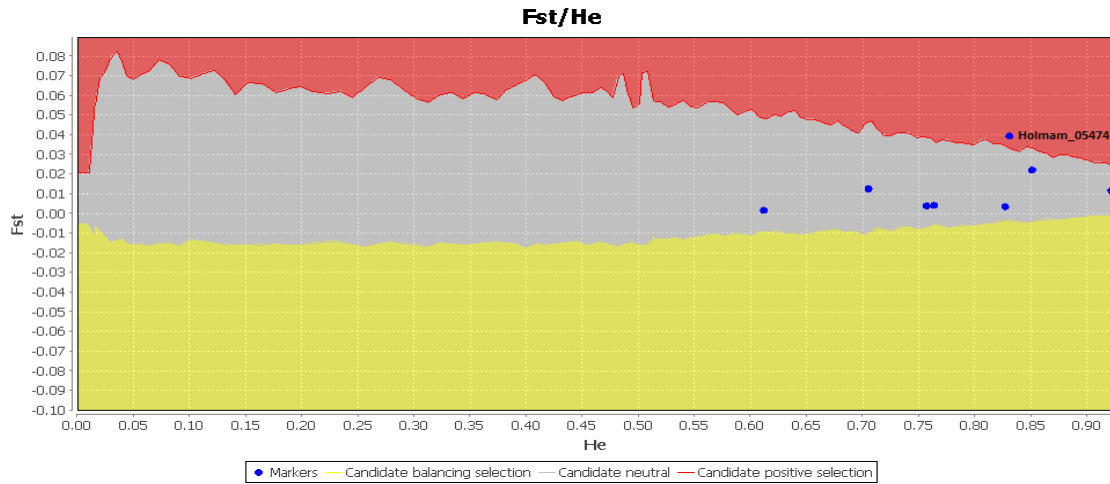


Figure 14 - Comparison of F_{ST} and H_e in polymorphic loci of *Holothuria mammata* to identify outliers and potential candidates for selection using LOSITAN software under the SMM. Graphical output shows the simulated confidence area for neutral loci (pale grey shading), positive selection (red area) and balancing selection (yellow bottom area). Loci outliers are tagged with labels. Locus Holmam_05474 is candidate for positive selection

4.2. Genetic Structure

The pairwise F_{ST} estimates for the 8 populations (Table VII), ranged from 0 (between Olhos de Água & Mallorca and Murcia & Mallorca) to 0.030 (between Kusadasi and Gran Canaria). Significant differentiation (F_{ST}) was found between Kusadasi and all sampling localities. Girona was also significantly different when compared with all populations excepting with Mallorca ($F_{ST}=0.009$; $p=0.095$), that only showed significant differentiation with Peniche and Kusadasi (Table VII). Murcia presented significant differentiation with Gran Canaria and Peniche from Atlantic Ocean, and with Girona and Kusadasi from Mediterranean Sea.

Although we found positive values of pairwise F_{ST} , no significant differentiation was found between Atlantic sampling localities ($p>0.12$). After sequential *Bonferroni* correction, significant differentiation was only found when comparing Kusadasi (Eastern Mediterranean) with all Atlantic Sea localities, and Girona with Peniche, Faro and Gran Canaria (Table VII).

Table VII: Pairwise fixation indices (F_{ST}) between 8 sampling localities of *Holothuria mammata* (below diagonal) and respective p-values (above diagonal).

Localities	PE	OA	FA	GC	MU	MA	GI	KU
Peniche (PE)	-----	0.117	0.141	0.393	0.021*	0.039*	0.000*	0.000*
Olhos de Água (OA)	0.008	-----	0.706	0.175	0.462	0.866	0.011*	0.000*
Faro (FA)	0.007	0.001	-----	0.529	0.300	0.105	0.000*	0.000*
Gran Canaria (GC)	0.007	0.011	0.003	-----	0.033*	0.131	0.000*	0.000*
Murcia (MU)	0.012	0.003	0.004	0.015	-----	0.985	0.039*	0.003*
Mallorca (MA)	0.011	0.000	0.008	0.011	0.000	-----	0.095	0.013*
Girona (GI)	0.023	0.017	0.024	0.025	0.011	0.009	-----	0.002*
Kusadasi (KU)	0.027	0.020	0.024	0.030	0.014	0.012	0.014	-----

* F_{ST} with significant values ($P<0.05$). Significant values after sequential *Bonferroni* correction are indicated in bold.

Since, *H. mammata* is present in the Mediterranean Sea and Atlantic Ocean, we tested if both regions (considering a larger spatial scale) are showing significant genetic differentiation such as it was demonstrated by other marine species. Our results (Table VIII) showed low but highly significant genetic differentiation among regions.

Table VIII- Pairwise fixation indices (F_{ST}), (below diagonal) and respective p-values (above diagonal).

Regions	Atlantic Ocean	Mediterranean Sea
Atlantic Ocean	-----	0.0000*
Mediterranean Sea	0.0097	-----

* F_{ST} with significant values ($P < 0.05$). Sign. values after sequential *Bonferroni* correction are indicated

Pairwise estimates of genic and genotypic differentiation were also calculated (Table IX). Twenty-six of 28 pairwise genic comparisons (92.8%) were significantly different across all loci. The genic differentiation was not significant between two population pairs: Faro with Gran Canaria and Murcia with Mallorca. These same population pairs did not presented significant genotypic differentiation. Genotypic differentiation tests presented less significant differentiation (53.5%) than genic tests. Both genic and genotypic differentiation (highly significant with $p < 0.001$) was found between: 1) Kusadasi with all Atlantic populations (*i.e.* Faro, Gran Canaria, Olhos de Água and Peniche); 2) Girona with all Atlantic populations; 3) Peniche with Mallorca. When considering both genic and genotypic differentiation, Kusadasi was significantly differentiated from all Western Mediterranean localities (MU, GI, MA). Some close Mediterranean localities, such as Mallorca and Girona, were both genic and genotypic differentiated. Genic and genotypic differentiation between some Atlantic and Mediterranean localities was also detected, such as Faro with Mallorca, Peniche and Murcia. No genotypic differentiation was registered between Atlantic populations.

Table IX-Total genic and genotypic differentiation between populations.

Population pair	Genic		Genotypic	
	χ^2	P-value	χ^2	P-value
PE & OA	53.1819	0.0000***	24.1975	0.1486
PE & FA	35.9686	0.0071**	18.8824	0.3990
OA & FA	34.0469	0.0124*	16.7399	0.5410
PE & GC	32.3995	0.0197*	14.1606	0.7185
OA & GC	51.5729	0.0000***	24.6982	0.1334
FA & GC	26.3385	0.0922	14.7107	0.6817
PE & CC	∞	0.0000***	38.1237	0.0037**
OA & CC	43.6410	0.0006***	22.6735	0.2034
FA & CC	41.9650	0.0011**	21.8335	0.2394

GC & MU	45.2581	0.0003***	26.0105	0.0995
PE & MA	∞	0.0000***	∞	0.0000***
OA & MA	43.1733	0.0007***	21.7408	0.2436
FA & MA	∞	0.0000***	36.8643	0.0054**
GC & MA	45.1418	0.0003***	26.0245	0.0991
MU & MA	25.0501	0.1235	13.0399	0.7891
PE & GI	∞	0.0000***	∞	0.0000***
OA & GI	65.1538	0.0000***	43.1641	0.0007***
FA & GI	∞	0.0000***	∞	0.0000***
GC & GI	79.4786	0.0000***	48.0274	0.0001***
MU & GI	43.4457	0.0006***	25.1404	0.1210
MA & GI	50.0020	0.0000***	29.8102	0.0393*
PE & KU	∞	0.0000***	∞	0.0000***
OA & KU	83.8830	0.0000***	67.6200	0.0000***
FA & KU	∞	0.0000***	∞	0.0000***
GC & KU	∞	0.0000***	53.8369	0.0000***
MU & KU	49.1747	0.0001***	31.2676	0.0268*
MA & KU	55.0049	0.0000***	37.2653	0.0048**
GI & KU	57.7138	0.0000***	41.9264	0.0011**

P-value for each population pair across all loci (Fisher's method). Significance of p codes: *0.01<p<0.05; **0.001<p<0.01;***p<0.001

The STRUCTURE software (Figure 15) identified two genetically differentiated clusters corresponding to Atlantic and Mediterranean regions, as the peak in \hat{K} was for $K=2$. These populations are consistent with the F_{ST} values obtained. A gradient of admixture between populations is observed, being this gradient lower in Eastern Mediterranean region (Kusadasi, KU). Considering the Atlantic localities, Olhos de Água (S Portugal) is showing the higher Mediterranean admixture.

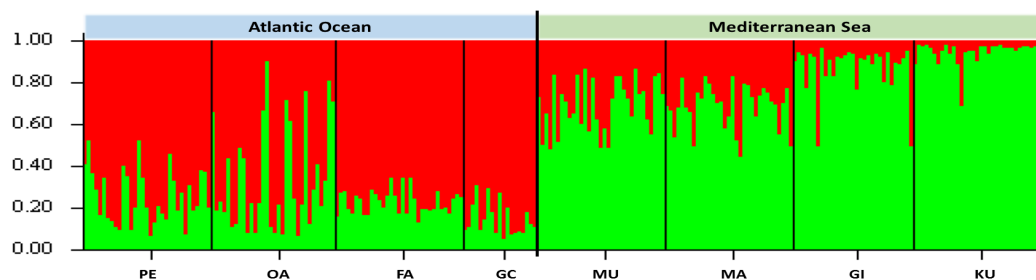


Figure 15 - Summary plot of estimates of Q at $K=2$. Each individual is represented by a single vertical line broken into K coloured segments, with lengths proportional to each of the K inferred clusters. The abbreviations correspond to the predefined populations.

The pattern of differentiation between sampled localities was studied through Mantel correlation to establish the relationships among F_{ST} values and

geographic/oceanographic distances (Table X). The correlations presented significant positive values describing a pattern where higher genetic differentiation is observed between samples located to larger geographic and oceanographic distances (Figure 16). When Kusadasi is not included in these analyses, the correlation between geographic distance and F_{ST} values decreases and loses significance, however the correlation using oceanographic distances improves.

Table X : Mantel test coefficient of correlation (r) and respective p-values between genetic (*i.e.* F_{ST}) and geographic/oceanographic distances of *Holothuria mammata*

Mantel Test (10000 permutations)	Geographic distances (km)		Oceanographic distances (km)	
	All Populations	Without KU	All Populations	Without KU
r (Pearson coefficient)	0.773	0.608	0.764	0.820
P-value	0.000*	0.056	0.000*	0.004*

* Coefficient of correlation with significant values ($P < 0.001$).

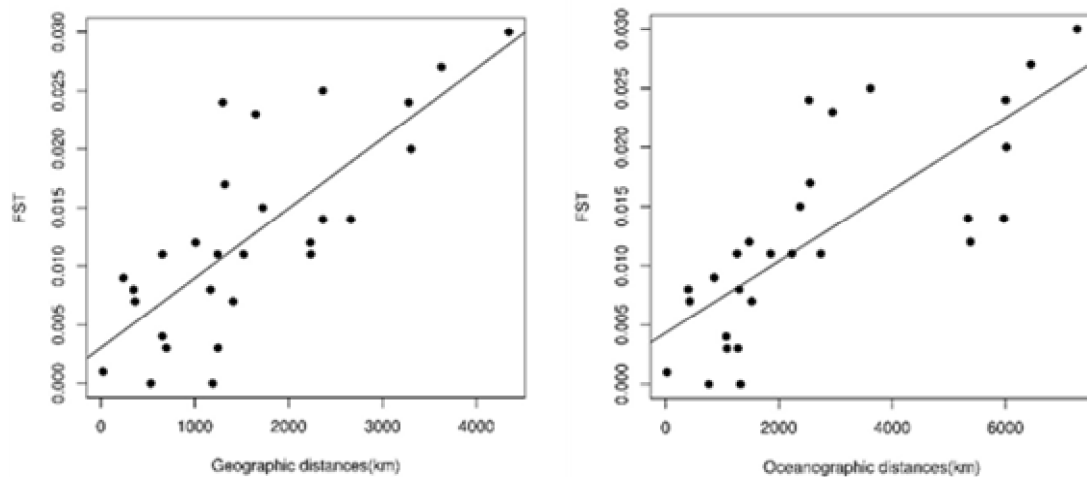


Figure 16- Relationship between pairwise F_{ST} and Geographic /Oceanographic distances for the 8 populations of *H. mammata*.

To verify the genetic structure patterns previously described, analysis of molecular variance considering different groups (Table XI) were carried out (see Methodology section).

The first grouping of sampling localities was justified by F_{ST} values considering three groups: Atlantic (PE, OA, FA, GC), south western Mediterranean (MU, MA) and other Mediterranean localities (GI, KU). This revealed low but significant percentage of variation among groups (0.91%; $p=0.000$) and high and significant variation within populations (98.48%; $p=0.009$).

When considering two groups as Island vs. Continental populations, hardly any variance was explained by the groups and most of the variation was within populations.

Considering the localization of the samples according to the edges of distribution of *H. mammata* two groups were considered: a first one, harbouring the samples located on edges of distribution, PE (North-western) and GC (South-Western) and a second one including the rest of localities (OA, FA, MU, MA, GI, KU). Small but significant differentiation was found among groups (0.56%; $p=0.000$), and the variation among populations within groups was also significant (1.06%; $p=0.000$).

The clustering of localities belonging to Atlantic (PE, OA, FA, GC) and Mediterranean (MU, MA, GI, KU) groups, found significant differentiation

Finally, three groups according to biogeographic criteria were established: Atlantic (PE, OA, FA, GC), Western (MU, MA, GI) and Eastern Mediterranean (KU). 0.97% of the variance was attributed to differences among groups, 0.60% among populations within groups, but most of the variance (98.43%) was significantly attributed within populations.

Table XI Analysis of molecular variance (AMOVA) among different groups setup.

Groups	Hierarchical level	Var. %	Fixation indices	P value
αAtlantic (PE, OA, FA, GC)	Among groups	0.91	$CT = -0.015$	0.000*
αSW Mediterranean (MU, MA)	Among populations within groups	0.61	$SC = -0.006$	0.109
αOther Mediterranean (GI, KU)	Within populations	98.48	$ST = -0.009$	0.009*
αIsland Populations (GC, MA)	Among groups	-0.38	$CT = 0.01$	0.000*
αContinental Pop. (PE, OA, FA, MU, GI, KU)	Among populations within groups	1.42	$SC = -0.014$	0.000*
	Within populations	98.96	$ST = -0.003$	0.931
αEdge Populations (PE, GC)	Among groups	0.56	$CT = -0.016$	0.000*
αCentral Populations (OA, FA, MU, MA, GI, KU)	Among populations within groups	1.06	$SC = -0.010$	0.000*
	Within populations	98.38	$ST = -0.005$	0.070
αAtlantic (PE, OA, FA, GC)	Among groups	0.76	$CT = -0.015$	0.000*
αMediterranean (MU, MA, GI, KU)	Among populations within groups	0.84	$SC = -0.008$	0.005*
	Within populations	98.40	$ST = -0.007$	0.027*
αAtlantic (PE, OA, FA, GC)	Among groups	0.97	$CT = -0.015$	0.000*
αW Mediterranean (MU, MA, GI)	Among populations within groups	0.60	$SC = -0.006$	0.004*
αE Mediterranean (KU)	Within populations	98.43	$ST = -0.009$	0.004*

*Significant values ($p < 0.05$)

Principal Component Analysis (Figure 17) based on the allele frequencies showed two main groups: Atlantic localities on positive side of Component I and Mediterranean samples in the negative side. Kusadasi belonging to this last group was located on the negative side of both components and differentiated to the other Mediterranean localities grouped on the positive part of component II. Both components (I and II) explained 40.8% of the variance.

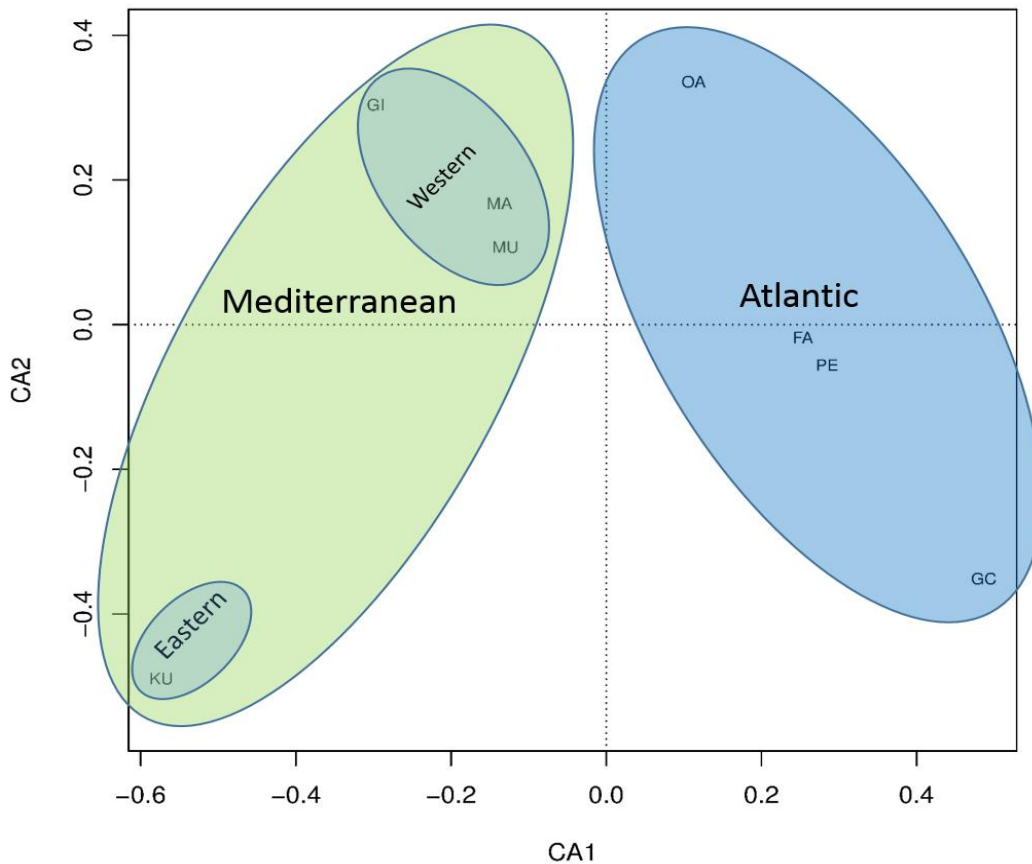


Figure 17 - Correspondence analysis plot based on the allele frequencies for the 8 populations of *H. mammata*.

4.3. Genetic Connectivity and Effective Population Size

Assignment tests (Figure 18) revealed a general pattern of high gene flow between Atlantic populations and consequently a low percentage of individuals were assigned to the population where they were collected (geographic origin), except for Gran Canaria with a 32% percentage assigned to the origin population showing a high self-recruitment. Olhos de Água had assignments to all sampled localities and Peniche and Faro to 7 locations. Olhos de Água and Peniche showed a high percentage of assignment to Faro with 25% and 28% respectively.

In the Mediterranean region, Girona and Kusadasi showed the greatest percentage of individuals assigned to their own localities (*i.e.* 32% and 46%), meaning lower flow of migrants with another locations and more self-recruitment. However, it is important to stress the geographic distance between Kusadasi and any other of the Mediterranean sampling localities. In Murcia (SW Mediterranean) similar assignments were found with all localities except for Olhos de Água (assignments not registered).

Interestingly, 34% of Mallorca individuals (SW Mediterranean) were assigned to Olhos de Água (S Portugal).

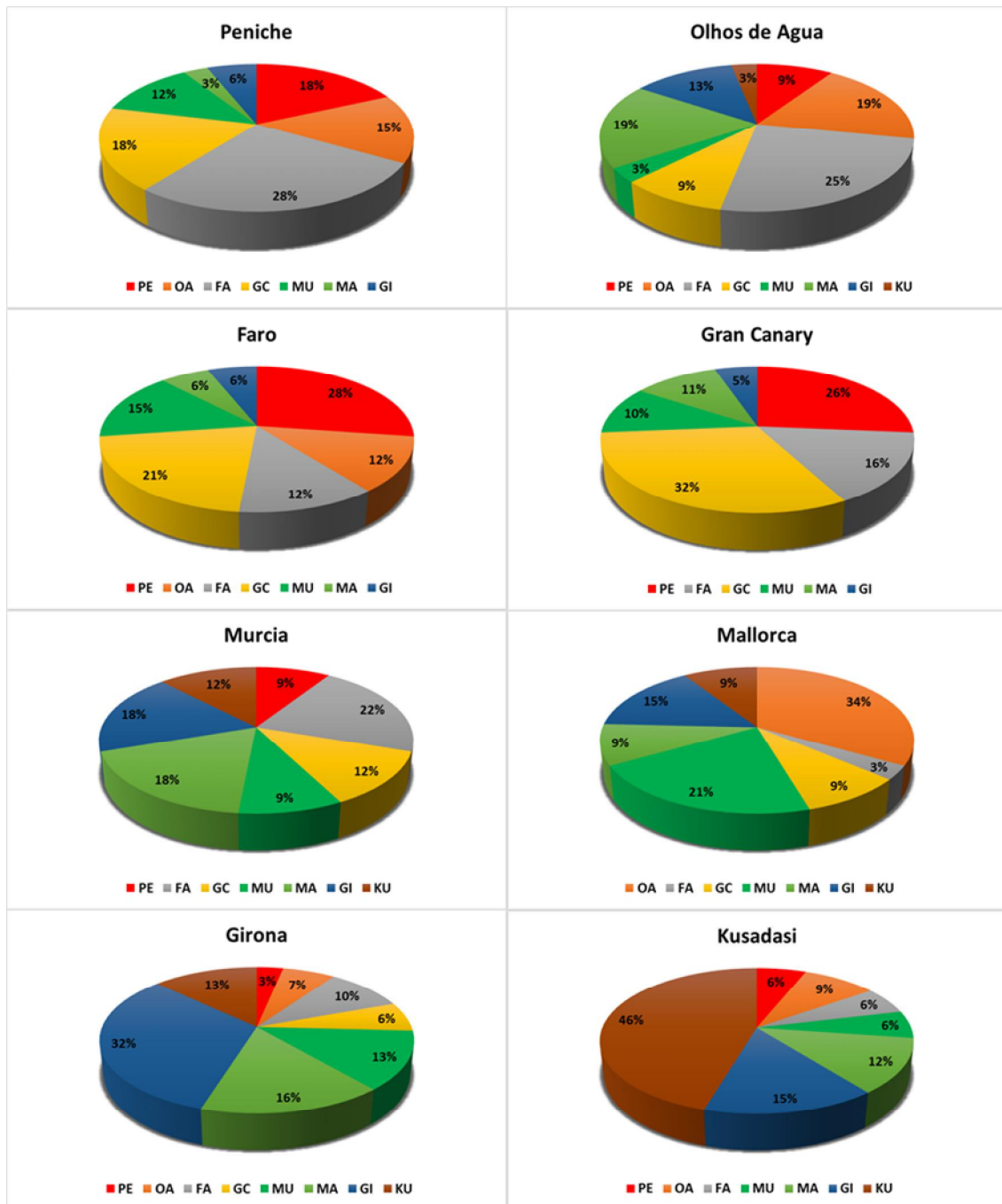


Figure 18 - Assignment tests of *H. mammata* individuals based on 10 microsatellite loci. The circular charts indicate the adjusted mean probability of the assigned individual actually belonging to a given population, based on the exclusion-simulation test.

Effective population sizes obtained from MIGRATE analysis (Figure 19), showed that Kusadasi and Girona have the highest effective population sizes and Gran Canaria and Murcia the lowest ones.

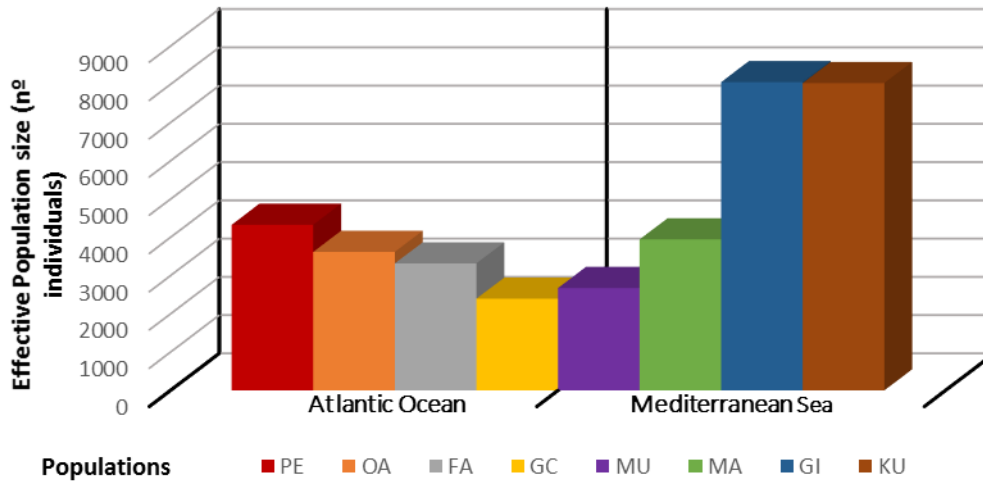


Figure 19- Column chart for each sampling location, showing the mutation scaled estimate of the effective populations size (N_e) for each sampling locality.

MIGRATE results (Table XII) allowed us to identify the main sources and sinks between our sampled populations. In general, migration rates were higher between Atlantic locations than in Mediterranean ones, although the maximum migration rate was found between Kusadasi and Murcia (16.6).

The main remarking results from the analysis with MIGRATE are:

- 1-High gene flow from Peniche to Olhos de Água and Faro.
- 2-Mallorca could be a sink population from Peniche, Faro, Olhos de Água and Murcia, although Murcia and Olhos de Água are acting simultaneously as source and recipient between them, with high gene flow.
- 3-High genetic exchange from Olhos de Água to Gran Canaria, which could be influenced due to small sample size of Gran Canaria.
- 4-Girona and Kusadasi were the populations with the lowest exchange of migrants, except from Kusadasi to Murcia.
- 5-Olhos de Água is showing a higher connectivity with SW Mediterranean (Murcia and Mallorca) than Atlantic region (Faro or Peniche).

Table XII-Migration rates ($M = m'$) based on 9 microsatellites loci of *Holothuria mammata* from sources (columns) to sink populations (rows).

Locality	Migration rates							
	PE to	OA to	FA to	GC to	MU to	MA to	GI to	KU to
Peniche	----	4.6	4.1	2.7	2.8	5.3	2.8	2.9
Olhos de Água	12.9	----	3.4	11.9	8.8	4.8	7.2	6.4
Faro	12.3	4.9	----	5.0	4.5	4.4	6.6	5.3
Gran Canaria	4.5	12.4	5.1	----	4.2	6.7	6.0	8.6
Murcia	4.9	8.1	5.5	2.6	----	6.8	8.6	16.6
Mallorca	12.8	7.8	9.3	4.2	9.9	----	2.2	1.8
Girona	1.1	1.8	1.9	2.4	1.0	1.3	----	2.4
Kusadasi	1.5	2.7	2.6	3.3	4.9	1.8	2.2	----

4.4. Bottlenecks

Tests for signatures of recent population bottlenecks (Table XIII) did not find any significant evidence considering Infinite Allele Model (IAM). However, Peniche, Faro, Gran Canaria and Murcia presented significant values for Wilcoxon and Sign tests under Two Phase Model (TPM), and Olhos de Água showed only significance for the Wilcoxon test under TPM.

Table XIII:- Heterozygosity excess/deficiency under different mutation models in all populations and respective Sign and Wilcoxon tests.

		Nº of locus with:	PE	OA	FA	GC	MU	MA	GI	KU
Infinite Allele Model (IAM)	Sign Test	Expected He excess	5.47	5.44	5.45	5.44	5.47	5.44	5.44	5.47
		He deficiency	4	5	6	6	6	5	4	5
		He excess	5	4	3	3	3	4	5	4
		Probability	0.498	0.258	0.092	0.093	0.091	0.256	0.506	0.251
	Wilcoxon test	P (One tail for He deficiency)	0.285	0.410	0.101	0.101	0.179	0.751	0.589	0.410
		P (One tail for He excess)	0.751	0.632	0.917	0.917	0.849	0.285	0.455	0.632
		P (Two tail for He excess and deficiency)	0.570	0.820	0.203	0.203	0.359	0.570	0.910	0.820
Two Phase Model (TPM)	Sign Test	Expected He excess	5.35	5.36	5.41	5.36	5.37	5.34	5.36	5.35
		He deficiency	8	6	7	7	7	6	6	6
		He excess	1	3	2	2	2	3	3	3
		Probability	0.004	0.104	0.024	0.026	0.026	0.107	0.104	0.105
	Wilcoxon test	P (One tail for He deficiency)	0.009	0.024	0.006	0.009	0.013	0.064	0.082	0.064
		P (One tail for He excess)	0.993	0.981	0.995	0.993	0.990	0.975	0.935	0.975
		P (Two tail for He excess and deficiency)	0.019	0.048	0.013	0.019	0.027	0.128	0.164	0.128

Parameters for T.P.M: Variance = 30.00; Proportion of SMM in TPM = 70.00%; Estimation based on 100 000 interactions; He: Heterozygosity. Bold values are significant at $P < 0.05$.

4.5. Morphometry Analyses

A total of 298 sea cucumbers were collected and measured in eight localities from Northeastern Atlantic Ocean and Mediterranean Sea. In total, length ranged from 9 cm to 43 cm and eviscerated weight from 17 gr to 235 gr. *Holothuria mammata* showed an average of length and weight of 21.07 ± 4.81 cm and 87.41 ± 40.79 g respectively (Table XIV).

Peniche was the locality showing the biggest individuals reaching values of 24.09 cm and 160.84 g. The lowest length and weight averages were recorded in Murcia, with values of 18.44 cm and 57.25 g respectively.

Table XIV: Summary of the data used in Length-Weight relationship for *Holothuria mammata*. (N: number of individuals sampled; STD: standard deviation; Min: minimum value; Max: maximum value.

Localities	N	Length (cm)			Weight (g)		
		Average \pm STD	Min	Max	Average \pm STD	Min	Max
Gran Canaria	18	19.78 \pm 4.04	14.0	27.0	66.83 \pm 24.28	27.0	128.0
Peniche	32	24.09 \pm 6.83	13.0	43.0	160.84 \pm 52.51	55.0	235.0
Olhos de Água	28	20.00 \pm 3.24	9.0	27.0	64.00 \pm 19.59	24.6	98.8
Praia de Faro	35	23.01 \pm 4.93	14.0	33.0	92.59 \pm 31.62	34.0	162.0
Murcia	52	18.44 \pm 4.55	9.0	38.0	57.25 \pm 20.33	17.0	104.0
Mallorca	52	21.38 \pm 3.32	13.0	33.0	84.69 \pm 26.55	36.0	150.0
Girona	31	22.39 \pm 4.94	15.0	34.0	96.10 \pm 30.91	43.0	175.0
Kusadasi	50	20.81 \pm 3.81	15.0	36.0	85.97 \pm 21.41	54.0	172.0
Total	298	21.07 \pm 4.81	9.0	43.0	87.41 \pm 40.79	17.0	235.0

Length and Weight frequencies distribution revealed a unimodal pattern for *H. mammata* (Figure 20 & 21). However considering regions, unimodal frequency distribution was observed on Mediterranean localities and a multimodal pattern on Atlantic ones (Figures 22 and 23). In general specimens from Atlantic, showed higher frequencies of the larger length classes than Mediterranean ones.

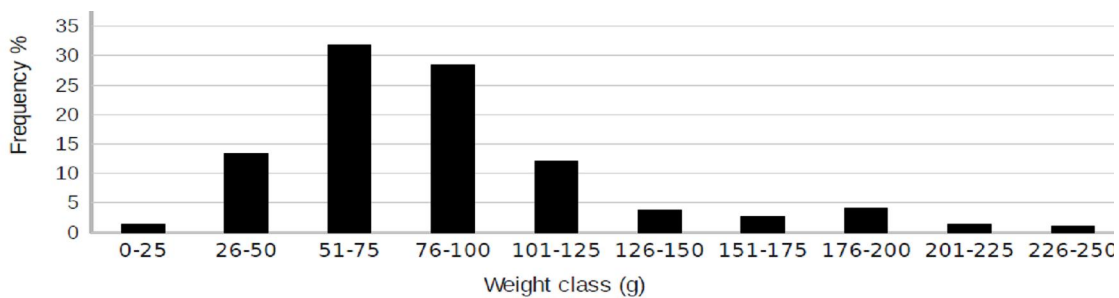


Figure 20- Weight - Frequency distribution of *Holothuria mammata* along all sampling locations

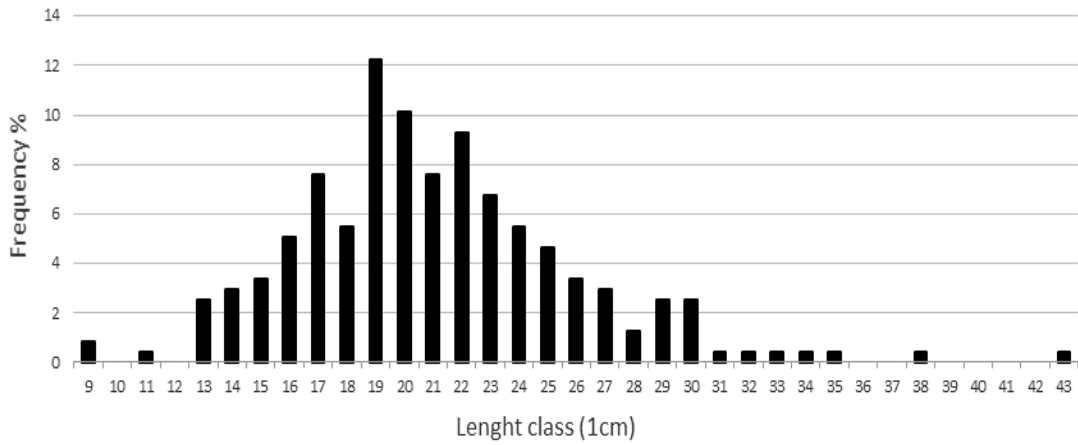


Figure 21 ó Length - Frequency distribution of *Holothuria mammata* along all sampling locations

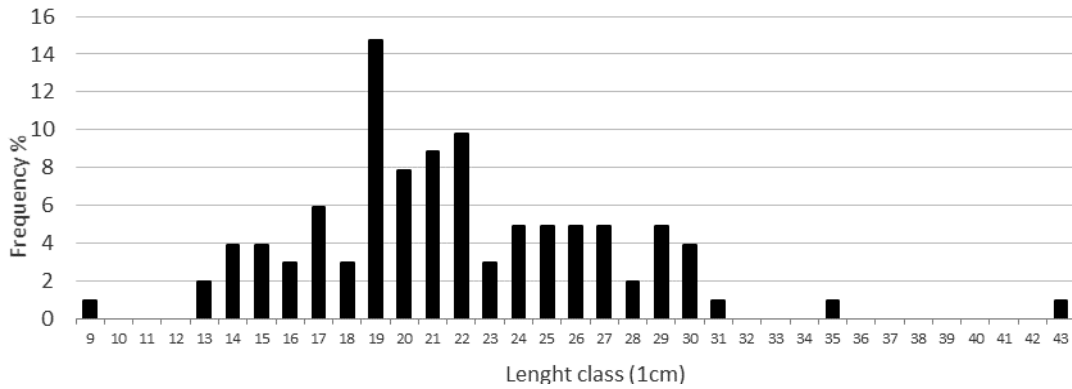


Figure 22 - Length-Frequency distribution of *Holothuria mammata* in Atlantic Ocean sampling localities

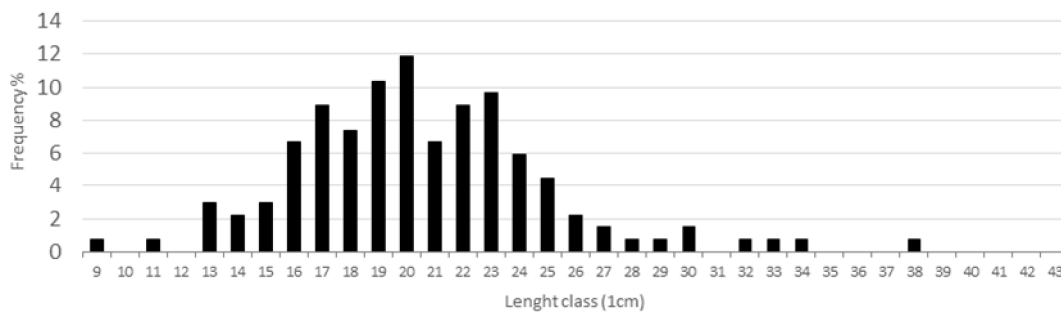


Figure 23 - Length-Frequency distribution of *Holothuria mammata* in Mediterranean Sea sampling localities

The differences between localities were highly significant ($p < 0.001$) on weight and length according ANOVAs analysis (Table XV; Figures 24 & 25), showing Peniche and Faro the largest and heaviest individuals. Among regions, the differences on length were significant ($p = 0.02$) and highly significant on weight ($p < 0.001$) (Table XV, Figure 26) with the biggest individuals belonging to Atlantic region.

Table XV- ANOVA considering the $\bar{\sigma}$ Locality $\bar{\sigma}$ and $\bar{\sigma}$ Regions $\bar{\sigma}$ as factors.

Factor	Variable	Sum-of-squares	df	Mean square	F- ratio	Pr(>F)
Localities	Length	963	7	137.57	6.749	2.02e-7***
	Weight	243031	7	34719	40.7	<2e-16***
Regions	Length	122	1	122	5,36	0.0213*
	Weight	32618	1	32618	20.92	7.06e-06 ***

Significance of p codes:0***,0.001**, 0.05*.

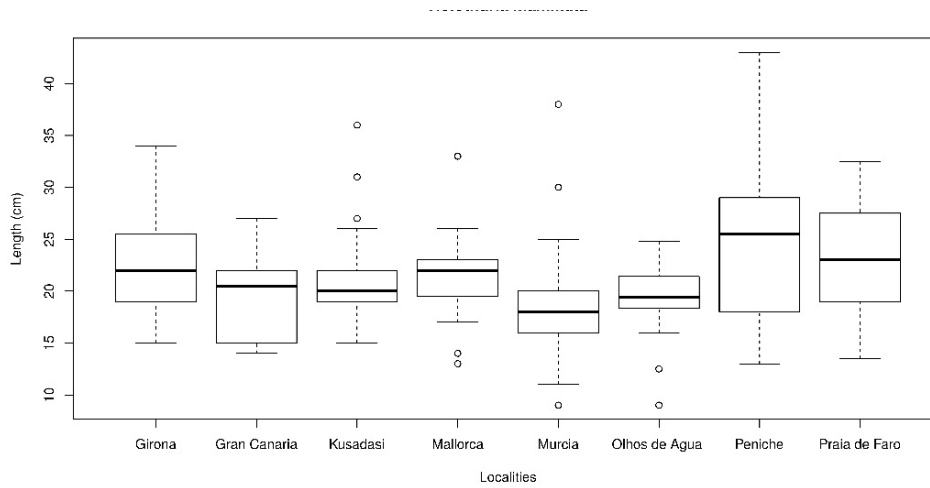


Figure 24 - Mean length in all sampled populations

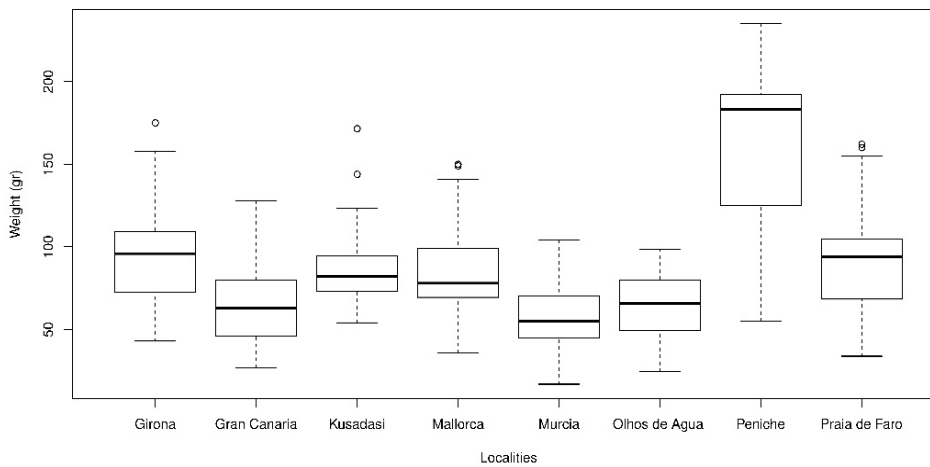


Figure 25 - Mean weight in all sampled populations

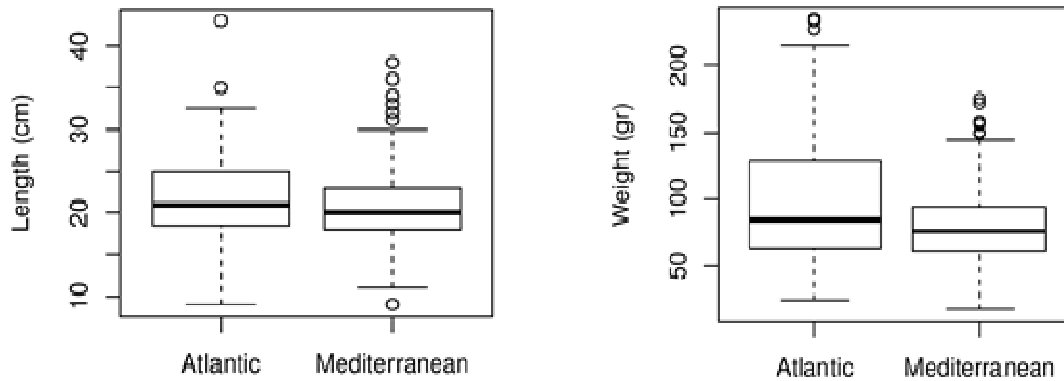


Figure 26 - Mean length and weight considering Atlantic Ocean and Mediterranean Sea populations

Pairwise comparisons after *Bonferroni* correction (Table XVI) revealed that Peniche specimens were significantly ($p=0.000$) heavier than individuals from all the others localities. However, the length of individuals from Peniche was not significantly different ($p>0.05$) of individuals from Girona, Mallorca and Faro, the locations showing the highest size averages. Murcia having the lowest average size and weight, revealed significant differences on weight with all localities except for Olhos de Água and Gran Canaria showing both lighter individuals. Only highly significant differences ($p=0.000$) on length were detected between Murcia and Peniche/Faro.

Table XVI-Pairwise comparisons using *t* test. *Bonferroni* *p*-value adjustment method was applied. Below diagonal Weight, above diagonal Length. Significant *p*-values are in bold.

Pop.	Girona	Gran Canaria	Kusadasi	Mallorca	Murcia	Olhos de Água	Peniche	Faro
GI	-----	1	1	1	0.003	0.266	1	1
GC	0.022	-----	1	1	1	1	0.036	0.395
KU	1	0.497	-----	1	0.217	1	0.042	0.795
MA	1	0.731	1	-----	0.027	1	0.217	1
MU	0.000	1	0.000	0.000	-----	1	0.000	0.000
OA	0.001	1	0.050	0.086	1	-----	0.001	0.038
PE	0.000	0.000	0.000	0.000	0.000	0.000	-----	1
FA	1	0.072	1	1	0.000	0.004	0.000	-----

Linear regression analysis revealed a highly significant length-weight relationship ($p<0.001$) on *H. mammata*, although the R-squared ($R^2=0.414$) was low. The b value was significantly different from 3, showing a positive allometric value of $b=5.61$ (Figure 27 and Table XXII).

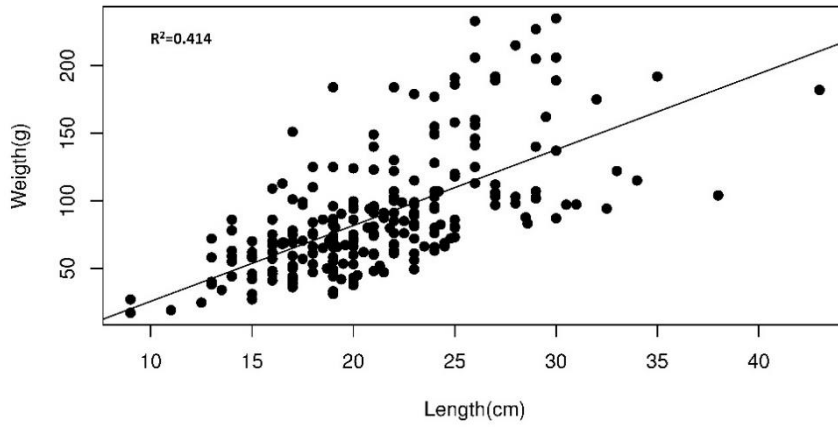


Figure 27 - Length-weight relationships and R-squared.

Table XVII: Coefficients of the linear model (EW vs. FW) for *Holothuria mammata*.

Coefficients	Estimate	Std. Error	<i>t</i> value	<i>Pr(> t)</i>
Intercept (a)	-30.5131	9.2549	-3.297	0.00112 **
Length (b)	5.6094	0.4264	13.155	<2e-16 ***

Significance of p codes: 0***,0.001**.

5. Discussion

This Master thesis provides the first population genetic assessment carried out with novel polymorphic markers (microsatellites) to the commercially valuable species, *Holothuria mammata*. This study considers different spatial scales covering nearly the geographical distribution of the species.

5.1. Genetic Diversity

Holothuria mammata showed high genetic diversity (*i.e.* allelic diversity, private alleles, H_e) in all populations. The comparison of these values with another holothurian species is difficult considering that until now no population genetics studies using microsatellites were published on European holothurians species. Only, some studies using microsatellites, were published on tropical species such as *Stichopus japonicus* (Chang *et al.*, 2009; Kang *et al.*, 2011). This species showed lower mean number of alleles and less observed heterozygosity than values registered on *H. mammata*. *S. japonicus* has been heavily overfished in the last decades, which could be explaining its lower genetic diversity.

A previous study focused on *Holothuria mammata* (Borrero-Pérez *et al.*, 2011), using mtDNA markers (COI and 16S genes) detected also high genetic diversity (16S gene: $H=0.93$; $\pi=0.006$; COI gene: $H=0.92$; $\pi=0.007$) on most of the populations covering the geographical distribution of the species. *Holothuria arguinensis*, a very close species of *H. mammata* distributed in NE Atlantic and colonizing the SW Mediterranean Sea, presented lower values of genetic diversity on mtDNA genes (16S gene: $H=0.88$; $\pi=0.005$; COI gene: $H=0.90$; $\pi=0.01$), perhaps due to its more restricted geographical distribution (Rodrigues *et al.*, 2015). *Holothuria polii* another species belonging to the *Holothuria* Genus and having overlapped its distribution with *H. mammata* in the Mediterranean Sea, showed lower values of genetic diversity for 16S genes (16S gene: $H=0.65$; $\pi=0.003$) than *H. mammata*, although similar results were obtained for COI gene (COI gene: $H=0.93$; $\pi=0.005$) (Valente *et al.*, 2015)

Considering the spatial scale of regions, our study on *H. mammata* using microsatellites found higher genetic diversity (number of total alleles, private alleles and observed and expected heterozygosity) on Mediterranean region than the Atlantic one. This pattern of diversity was already described by Borrero-Pérez *et al.*, (2011) on *H.*

mammata using COI gene, however their results obtained from 16S gene did not corroborated it.

Similar results were found for other invertebrates, like equinoderms (Pérez-Portela *et al.*, 2010) and crustaceans (Roman & Palumbi, 2004) , however they attribute these differences in genetic diversity due to former colonization into the Atlantic Ocean or vice versa. Higher diversity in the Mediterranean region could be driven by more complex environmental conditions and historic climatic fluctuations than the Atlantic one. Important gradients of temperature, salinity and nutrients have been described for the Mediterranean Sea coupled with highly variable currents circulation (Danovaro *et al* 1999, Bethoux *et al* 1999, Pattarnelo 2007). This historical and contemporaneous heterogeneous environmental conditions might induce higher intraspecific variability enhancing the adaptive potential for adverse and changing conditions (Pamilo, 1988; Lowe *et al.*, 2012; Mitchell *et al.*, 2015) .

When smaller spatial scale (locations) is taken into account, Kusadasi (Turkey) and Murcia (SE Spain) presented the highest mean allelic diversity (13.7 and 14.2 respectively) and observed heterozygosity (0.71 and 0.61 respectively). In Murcia, the influence of different oceanographic regimes (*i.e.* Atlantic waters, Northern current) could increase its connectivity, receiving migrants from the nearby populations of Western Mediterranean and Atlantic. Murcia has high gene flow (*i.e.*, no differentiation) with Olhos de Água and Faro (S Portugal), but also with Mallorca (E Spain) and Girona (*i.e.* genotypic), which might contribute to its higher gene diversity. Kusadasi (Turkey) is influenced by the different hydrographic features of the two Aegean sea sub-basins (Ignatiades *et al* 2002) where the North area is showing a high primary productivity and the South area is an oligotrophic environment (Siokou-frangou *et al* 2002). Migrants from both sub basins, might contribute with different gene pools to the recipient Kusadasi population. However, this hypothesis must be considered with limitations, because only one locality from Eastern Mediterranean was studied. Further genetic studies, sampling several localities from this region could be necessary to confirm or disregard this hypothesis. Other factor could be considered to explain our results of high genetic diversity in Kusadasi, the existence of older lineages from putative glacial refugia located in this geographical area. However, this hypothesis does not seem to be supported by the previous phylogeographic study on *H. mammata* using mtDNA markers (COI and 16S genes) (Borrero-Pérez *et al.*, 2011) which found the lowest

genetic diversity in a Turkish population (Foça); but it is important to stress that this locality was under heavy fishery pressure which could affect the results found.

The lowest genetic diversity values were found in Gran Canaria (Atlantic), but this result could be biased partially by the low number of individuals sampled there. However, other reasons could be affecting the diversity found in Gran Canaria such as island isolation and its localization at the Southern edge of geographical distribution of *H. mammata* (Borrero-Pérez *et al.*, 2011). Populations inhabiting the edges of distribution usually show a low effective number of individuals suffering a founder effect with decrease of genetic diversity and few rare variants (Excoffier 2009). Finally, the high abundance of another sea cucumber species, *Holothuria sanctori*, on this locality (Navarro *et al.*, 2013) competing by the same resources, could affect the *H. mammata* population size and therefore its genetic diversity.

Heterozygote deficiency in relation to HWE and positive significant F_{IS} values were found in all populations of *H. mammata*. Significant and positive F_{IS} values were also found (although not in all loci) in populations of *S. japonicus* in both published studies using microsatellites (Chang *et al.*, 2009; Kang *et al.*, 2011). This deficit of heterozygotes could be explained by several causes such as laboratory artefacts, unidentified null alleles, loci under selection, non-random mating and Wahlund effect (Allendorf *et al.*, 2013). Results from INEST analysis on *H. mammata*, clearly indicated the null alleles as the most contributing factor for positive and significant F_{IS} values in most of the populations. Some studies have found that free-spawners producing millions of eggs could have higher mutation rates caused by larger numbers of cell cycles, contributing to an increase in the frequency of null alleles, due to mutations on the microsatellite primer binding sites (Addison & Hart, 2005). Since most sea cucumbers (including *Holothuria mammata*) are broadcast spawners, it would be possible the existence of null alleles in our populations due to higher rates of natural mutations on primers binding sites. Gran Canaria was the only place where INEST did not attributed null alleles as the main factor contributing to the HWE deviations found, which supports evidences for true inbreeding in this population. In Gran Canaria, one third of the individuals are potential descendants from local population, meaning an important local retention of larvae (self-recruitment), which could promote inbreeding on this population and deficit of heterozygosity. On the other hand, the effective population size on this location was the smallest one, favouring also the inbreeding and loss of genetic variation due to stronger effects of the stochastic genetic drift.

The hypothesis of sweepstakes reproductive success promoting inbreeding and low effective population sizes (Hedgecock *et al.*, 2007b) , could also explain the HW disequilibrium detected on our populations but further studies must be done to corroborate it.

5.2. Genetic Differentiation

5.2.1. Great Spatial Scale: Atlantic and Mediterranean Regions

The genetic analysis performed on *H. mammata* revealed genetic subdivision between Eastern Mediterranean (*i.e.* Kusadasi), Western Mediterranean and the NE Atlantic regions. At this great spatial scale, pairwise F_{ST} comparisons showed a significant subdivision and STRUCTURE analyses also confirmed clearly the genetic break between NE Atlantic Ocean and Mediterranean Sea, revealing a decreasing gradient of Atlantic pool admixture in Eastern direction. These results agree with the previous ones obtained for the same species by Borrero-Pérez *et al.*, (2011) using mtDNA markers (COI and 16S genes). These authors also found genetic breaks between populations from the North-Eastern Mediterranean Sea (*i.e.* Aegen Sea), West Mediterranean and NE Atlantic Ocean, although considered Algarve (*i.e.* FA) and West Mediterranean (*i.e.* MU&MA) as a panmictic metapopulation.

Differentiation between Atlantic and Mediterranean basins have been widely illustrated on other echinoderms, such as sea urchins (Duran *et al.*, 2004) which only disperse at the larval phase (*i.e.* Pelagic larval duration or PLD, between 20-40 days), molluscs, like limpets (Sá-Pinto *et al.*, 2012) which also disperse with pelagic larvae (PLD up to 31.5 days) or crustaceans, like the global invasive crab *Carcinus maenas* (Roman & Palumbi, 2004), which have a approx. 40 days of PLD (Mohamedeen & Hartnoll, 1990) . This Atlanto-Mediterranean genetic break pattern was also described in fishes, as the swordfish, with high dispersal ability during adult stage and able to perform long distance migrations (Vinas *et al.*, 2010). Some Sparids, closely related species, with similar ecological, behavioural and evolution, such as *Diplodus puntazzo* and *Diplodus sargus* also presented marked differences in the differentiation patterns between both basins, with the former presenting marked genetic differentiation and latter with no significant genetic divergence between Atlantic and Mediterranean, respectively (Bargelloni *et al.*, 2005).

At NE Atlantic spatial scale, a lack of genetic structure was found. Two hypothesis could explain these results. First one considers that Atlantic populations could be the result of an historical expansion from the Mediterranean Sea, in which due to a limited evolutionary time passed since the Atlantic colonization, populations did not have enough time to differentiate between them. This hypothesis is supported by bottleneck results, lower number of rare alleles in Atlantic than Mediterranean and lower genetic diversity. The second hypothesis is the existence of high gene flow and connectivity between Atlantic locations which hampers their genetic differentiation. The oceanographic features of this region are showing a predominant southward flow (but not exclusively) moving at relatively faster speed, connecting genetically the Atlantic locations which could be considered as a panmictic metapopulation. This high gene flow scenario between Atlantic localities as it was stressed previously, could prevent genetic differentiation, but at the same time increase the genetic diversity in the edges of *H. mammata* distribution.

According to our results, the Atlantic populations seem to be genetically connected with Southern-Western Mediterranean (*i.e.* Murcia and Mallorca), perhaps favoured by the constant Atlantic water inflow into the Alborán sea and seasonally in Balearic Sea (Renault *et al.*, 2012b; Sayol *et al.*, 2013a). However this Atlantic influence does not seems to reach the NW Mediterranean area (*i.e.* Girona). Similar results were found by Borrero-Pérez *et al.*, (2011) establishing the Algarve region (FA) and West Mediterranean (MU&MA) as a panmictic metapopulation.

5.2.2. Small Spatial Scale: Sampled Locations

The migration rates obtained from Migrate analysis should be considered carefully and taking into account the time scale of models and markers used. The coalescent approach combines the mutational-scaled immigration rates, meaning that the results (average migration rates) are a combination of historical and contemporaneous gene flow (Vitor C. Sousa *et al.*, 2011; Wilkinson-Herbots, 2012). Therefore, we only consider the migration rates and their directionality, when they are showing biological meaning and are congruent with the actual oceanographic patterns and with the results from the assignment tests. In this way, we could obtain a more accurate model of the actual source and sink dynamics on *H. mammata*.

It is important to stress that, the low F_{ST} values found between some locations should be interpreted with caution and to be considered another parameters assessing genetic structure such as genic and genotypic differentiation. The mathematical dependency of F_{ST} on the allele frequencies and diversities must be taken in account, particularly when the population presents high diversities (as *H. mammata*) which biases downwards the F_{ST} values. Genic and genotypic differentiation between populations could detect in these cases more accurately the subdivision patterns (Jakobsson *et al.*, 2012).

At location scale, the connectivity patterns are strongly influenced by the ocean currents, such as it was demonstrated from the results obtained by Mantel test using oceanographic distances. In our study, some localities presented significant genic/genotypic differentiation (*e.g.* Girona (NE Spain) and Mallorca (E Spain) separated by 240 km) while other ones, such as Olhos de Água (S Portugal) and Mallorca separated by 1000 km, showed a lack of differentiation (*i.e.* genotypic and F_{ST} values). In NW Mediterranean Sea, the existence of the predominant Northern Current flowing southward and the Atlantic water flowing northeastward are acting as a density barrier between Mallorca and Girona forming the Balearic Front (Robinson & Leslie, 2001), explaining why these localities have negligible genetic exchange (*i.e.* are isolated), in spite of being geographically close. This pattern of genetic isolation between Mallorca and the Northeastern coast of Spain, was also found in several fish species with different early life histories traits (*e.g.* egg type, pelagic larval duration), reinforcing evidences that oceanographic patterns play a bigger role in shaping connectivity at this spatial scale (Galarza *et al.*, 2009; Schunter *et al.*, 2011) than just ecological dispersal potential of species.

Murcia (SE Spain), in opposite way to Mallorca, seems to have some gene flow with Girona (*i.e.* supported by non-genotypic differentiation and migration rates) in spite of F_{ST} significant values, and high gene flow with the Atlantic populations. The Northern Current, which flows slowly (*i.e.* low kinetic energy) in only southward direction close to the Spanish coast, suggests Girona as probable source population. The Atlantic waters moving rapidly with high kinetic energy and periodically (*i.e.* in Mid-Summer, the AOF relaxes and AW moves through the Ibiza Channel), arrive to Murcia allowing a high gene flow from the Atlantic populations (*i.e.* source populations). Murcia in fact could be considered as a sink but also as source population because receives migrants from several sources depending on the oceanographic regime in this

zone (if WA dominates or if the AOF pushes AW to African Coast allowing to the Northern current to reach Murcia), and also send migrants to another locations such as Mallorca.

According our results, Girona has more local recruitment than immigrants arriving from the rest of sampled locations. However, the AW moving southward into a slower mesoscale gyre along African, Corsica and French coast in an anti-clock wise direction and eventually becoming the dense Northern current after travelling thousands of km, could suggest another source populations for Girona. However we cannot assess this hypothesis because no sampling was performed in those areas.

Kusadasi (SW Turkey) shows clearly an isolation by distance (IBD) that complicates the assessment of connectivity patterns with the rest of our sampling localities, since it was the only sampled population in the Eastern Mediterranean Sea. Due to this IBD pattern, the migration rates obtained for Kusadasi were the lowest ones detected, (except with Murcia) and the migration rates for this locality are probably related to a historical pattern of gene flow, rather than a contemporaneous exchange of migrants.

Peniche (W Portugal) could be considered as source of Olhos de Água and Faro (S Portugal) according to Migrate results. The oceanographic patterns in the Iberian Coast during the spawning season of *H. mammata* have an interannual variability circulation patterns which favours bidirectional North / South exchange of larvae at a interannual temporal scale, which could favour the connectivity between these locations.

Faro (S Portugal) is acting mainly as a sink population, receiving migrants from the different populations. The role of source for Faro population is not likely since *H. mammata* population inhabiting Ria Formosa is not very abundant considering that the sandy/muddy bottoms with seagrass is not suitable habitat for this species which prefers rocky bottoms (Borrero-Pérez *et al.*, 2011; Siegenthaler, 2013). This role is different to the previously observed for *Holothuria arguinensis* inhabiting Ria Formosa (Rodrigues *et al.*, 2015). These authors found evidences that Ria Formosa populations of *H. arguinensis* are contributing to the overall genetic diversity of the species acting as source. In that case, it was demonstrated that the populations of *H. arguinensis* in Ria Formosa have high effective population sizes and show high densities ranged from 140 to 563 ind ha⁻¹ (González-Wangüemert *et al.*, 2013; Siegenthaler *et al.*, 2015).

Olhos de Água (S Portugal) and Gran Canaria are acting simultaneously between them as source and sink. This pattern could be explained by the current system only with gene flow from Olhos de Água to Canaries, but not the inverse. This corroborates the fact that results between Canaries and the others populations must be considered carefully because they could be influenced by the small sample size of Gran Canaria. Olhos de Água is showing a higher connectivity with SW Mediterranean (Murcia and Mallorca) than Atlantic region (Faro or Peniche). According the oceanographic patterns for the Southern Iberian Coast (García-Lafuente *et al.*, 2006), Olhos de Água is more influenced by the Cyclonic eddy (*i.e.* core N1, figure 3) than Faro, which could result in increased larval export to Mediterranean. Also, Olhos de Água is more exposed to oceanographic conditions (*i.e.* the current that flows over the continental shelf break and slope) than the Ria Formosa coastal lagoon (Faro), increasing the chance of gene flow from Olhos de Água to Murcia and Mallorca.

5.3. Bottlenecks

Populations that have experienced a recent expansion of their effective population sizes exhibit a correlative increase of the allele number and heterozygosity at polymorphic loci. Usually, the allele number is raised faster than heterozygosity (H_e), therefore the H_e becomes smaller (*i.e.* deficiency) than the expected heterozygosity at mutation-drift equilibrium (Maruyama & Fuerst, 1984; Cornuet & Luikart, 1996; Piry *et al.*, 1999; Girod *et al.*, 2011).

Although detected only in the T.P.M model, significant signature of recent expansion after bottleneck or founder event was found in Peniche, Olhos de Água, Faro, Gran Canaria and Murcia meaning that these populations are not in a drift-mutation equilibrium. In the cases of Peniche and Gran Canaria, their localization in the edges of *H. mammata* distribution could explain their signature of found event.

Murcia, Faro and Olhos de Água have bottleneck signatures difficult to justify although anthropogenic factors (such as fishery catches) cannot be ruled out. Availability of optimal habitat area, or inter-annual variability of food resources in these areas could also affect the demography of these populations. However no clear evidences were found about reasons explaining the bottlenecks, and therefore further ecology studies should be done.

5.4. Morphometry

There is a generalised lack of information about length and weight frequencies for *Holothuria mammata*, except for Turkish region (González-Wangüemert *et al.*, 2014) and Canaries (Navarro *et al.*, 2013). This study compiles considerable amount of weight and length data from *Holothuria mammata*, covering most of its distribution area. Our results demonstrated that there is high variability in size and weight between locations along the distribution of the target species. This might be related to local nutrient regimes at each particular site (see below). However, significant trend was found with larger animals in the Atlantic than in the Mediterranean, and in the Western than in the Eastern Mediterranean.

The average size (21.07 ± 4.81 cm) of *H. mammata* is in general smaller than other commercial species of *Holothuria* genus from the Indo-pacific such as *H. scabra* (29.00 cm), *H. pulla* (30.90 cm), *H. atra* (32.00 cm), *H. nobilis* (27.00 cm) and *H. fuscogilva* (29.50) (Khalfan *et al.* 2007, Herrero-Pérezrul & Reyes-Bonilla 2008). However, sea cucumbers are soft body individuals with the ability of high body contraction which brings a common larger error on the length measurements due to the difficulty in obtaining the complete relaxation of the body muscle. Thus, the use of gutted weight measurements could be more accurate than length to estimate population dynamics as it was stressed by other authors (Conand 1981, Bulteel *et al.* 1992, Tuwo & Conand, 1992, Kazanidis *et al.* 2010).

In Peniche, *H. mammata* specimens presented marked and significant differences in weight with all others localities. In this location, Northerly winds favoring seasonal coastal upwelling which are observed especially during summer (June to September) (Peliz *et al.* 2002; Álvarez-Salgado *et al.* 2003), enhancing the availability of nutrients in this area. Moreover, this locality is also positioned at the top of the Nazaré Canyon, a topographic feature that penetrates the continental platform allowing a very close to shore upwelling and additional enrichment in this region (Mendes *et al.* 2011). A higher availability of nutrients could favor the faster growth of bacteria community and diatoms in the bottom allowing a higher size and weight of *H. mammata* individuals inhabiting this area.

Inversely, Gran Canaria and Murcia presents the lightest and shortest specimens of *H. mammata*. The seafloor bathymetry around Canaries is abrupt with narrow shelf and a steep slope to more than 1000 meters depth, producing near shore conditions

similar to open sea (Navarro-Pérez & Barton 2001). These features could limit the abundance of some species (Popescu & Gras 2013) such as *H. mammata*. Moreover, Canary Islands are located in the southern edge of distribution of *H. mammata* (Borrero-Pérez *et al.* 2009). Usually resources and conditions (abiotic and biotic) of these marginal habitats are not optimal for growing and reproduction (Kawecki 2008). In addition, *H. mammata* could compete for food and habitat with another sea cucumber species (*H. sanctori*) very abundant in Canary Islands, since both species have similar rocky bottoms preferences (Navarro *et al.* 2013)

In Murcia the low size and weight values are difficult to explain, since the abundance of *H. mammata* in this location was high. This population is inhabiting a rocky restricted area showing the optimal habitat for this species but surrounded by sand beaches, which could be affecting to the biological features of the species. Further ecology studies are necessary to better understand our morphometric results in this locality.

Girona was the Mediterranean locality with biggest specimens (*i.e.* weight and length). The sampling locality in Girona is influenced by sewage discharges (pers. observation) which can promote nutrient and organic matter enrichment of the marine environment (Guidetti *et al.* 2003, Vizzini & Mazzola 2006)□ The input of nutrients (mainly nitrates and phosphates) could stimulate algal growth and increase the input of organic matter to the sediment - water interface (Boesch 2002)□, also increasing heterotrophic bacteria that utilize organic carbon produced by algae (Amin *et al.* 2012). Both microalgae and bacteria are the main food sources for sea cucumbers (Uthicke 1999, 2001). Since sea cucumbers are deposit feeders which use the organic matter directly at the sediment surface, they are likely to benefit from the high food availability in this location, therefore reaching higher sizes (Amon & Herndl 1991a, b, Navarro *et al.* (2013).

Unimodal length and weight frequency distributions were obtained for *H. mammata*. Other studies involving another species of sea cucumbers such as *Actinopyga echinites*, *Stichopus variegatus*, and *Holothuria forskali* (Conand *et al.* 1982, 1993, Tuwo and Conand, 1992) also showed a unimodal length frequency-distribution. However multimodal length frequency distributions were obtained when we considered each sampling locality separately (data not showed). Mediterranean studies of *H. mammata* (González-Wangüemert *et al.*, 2014) confirms this same length frequency

distribution pattern in Turkish waters. The most probable causes explaining the different patterns of size distributions between localities could be due to differences on environmental features such as temperature, nutrient availability and competition for food resources due to the presence of other sea cucumber species.

Morphometric relationships followed a positive allometric growth for *H. mammata* ($b = 5.60$). This allometric positive growth was previously described for *H. mammata* in Turkish waters (González-Wangüemert *et al.*, 2014a), and it was also described as characteristic growth for most holothurian species (Ramon *et al.*, 2010). This strong positive relationship ($0.4 < r \text{ index} \leq 0.7$) between length and weight, was significantly different from isometric growth ($b \approx 3$) and highly correlated ($p < 2.2e-16$). Similar values of parameter b (*i.e.* $b > 3$) were registered on *H. mammata* from Turkey (González-Wangüemert *et al.*, 2014) suggesting that larger specimens increase in height or width more than in length. Accordingly to Froese (2006), this could result from two reasons: 1) ontogenetic changes in body shape, it could be rejected because as far as we know, sea cucumbers only suffer strong ontogenetic changes on body shape during larval development; 2) most common reason is the presence of larger individuals being thicker than smaller ones, meaning a better nutritional condition in larger specimens than in juveniles. Better condition on adults is expected, since they reduce their energy demands necessary for growth when reach the maturity. Adults life strategy implies energy storage, which is used for gonads maturation and better condition of their eggs, however juveniles use the energy for growth mainly (Reyes-Bonilla & Herrero-Pérezrul 2003, So *et al.* 2010, Poot-Salazar *et al.* 2014).

6. Conclusion

6.1. Populations Genetics

This study was the first population genetics evaluation using polymorphic microsatellites markers in order to compensate the lack of knowledge for this ecological important species, and to provide important data for future management and conservation.

Holothuria mammata showed great genetic diversity, and differentiation between Atlantic and Mediterranean basins. The gene flow within and between Atlantic Ocean and Mediterranean sea populations is greater when oceanographic conditions favors the exchange of larvae, connecting Atlantic populations with Mediterranean ones, and thus increasing the populations genetic variation. In Mediterranean the spatial scale of differentiation is smaller than the Atlantic, due to historical events and contemporaneous complex spatial patterns of oceanographic regimes.

6.2. Morphometry

In conclusion, this morphometric analysis allowed us to have a first insight into the length-weight classes and growth pattern of *Holothuria mammata*, a species with commercial interest. Length-weight classes and relationships have not been previously reported for the species in such a wide geographical distribution range and by which allowed biogeographic comparisons. Due to their high commercial value coupled with a high risk of stock depletion, management measures should be taken. Therefore, besides setting biometric baselines, these results could be useful to create new legislation as well as used as reference to compare between natural / protected and exploited populations in order to evaluate the fishery effects on these target species.

7. Recommendations for Fisheries and Conservation Management

Considering the results obtained in this Thesis, we suggest the following recommendations for management of *H. mammata* as a resource:

1. *Holothuria mammata* should be managed taking in account three distinct units/stocks: 1) the northeast Atlantic Ocean; 2) the western Mediterranean Sea; 3) in the eastern Mediterranean Sea. Management regulations should be aimed at this spatial scale in order to avoid overfishing of smaller discrete populations.

2. At small spatial scale (locations), the resource in Gran Canaria should not be harvested, due to small effective population size and low genetic diversity. Fisheries focused on these island populations should be avoided in order to impede further deterioration of the adaptive potential of the population.

3. Source populations in the NE Atlantic Ocean, especially in Peniche, should also be conserved in order to maintain the patterns of connectivity, and consequently larval spillover to southern populations of the Portuguese Coast. Moreover this population appears to live in an edge distribution limit, reinforcing their fragility to climatic oscillations and consequently to fisheries.

4. Ria Formosa (*i.e.* Faro) also presents a unique population (*i.e.* high diversity, high number of rare alleles), that should be conserved, so it can become well established and increase exportation of larvae to others sink populations such as Gran Canaria and Murcia, improving their gene pool.

5. In the Eastern Mediterranean Sea, the Kusadasi population should also be subject of a precautionary approach when implementing fisheries, since it is harboring the highest genetic diversity.

6. In the Western Mediterranean Sea, the Murcia populations is showing a small effective population size and the smallest size individuals. Therefore any catches should not be authorized in this location until the reasons explaining these features are understood.

8. References

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Webpage of the CUMFISH Project: <http://www.ccmr.ualg.pt/cumfish/>

9. Appendix

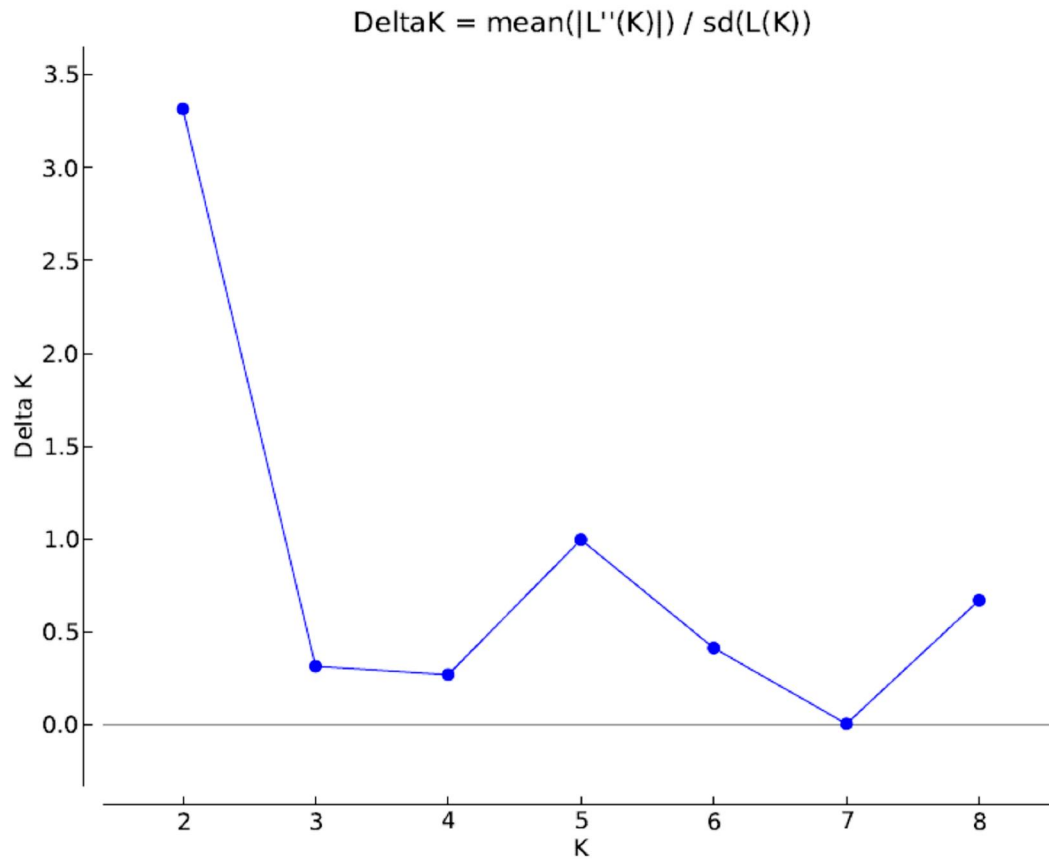


Figure 28-Magnitude of $\hat{e} K$ as a function of K (mean \pm SD over 10 replicates) for 9 microsatellites.

Table XVIII-Table output of the *Evanno* method results. Bold values shows the largest value in the Delta K column.

# K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	Ln''(K)	Delta K
1	10	-9672.95	0.4836	NA	NA	NA
2	10	-9645.75	31.9718	27.2	106.04	3.316676
3	10	-9724.59	115.3233	-78.84	36.34	0.315114
4	10	-9767.09	138.3375	-42.5	37.31	0.269703
5	10	-9772.28	228.4774	-5.19	227.68	0.99651
6	10	-10005.2	514.6602	-232.87	212.78	0.413438
7	10	-10025.2	551.3388	-20.09	2.72	0.004933
8	10	-10048.1	409.1386	-22.81	274.22	0.670237