ALMA MATER STUDIORUM
UNIVERSITA' DI BOLOGNA
FACOLTA' DI Scienze Matematiche, Fisiche e Naturali

Corso di laurea magistrale in *Biologia Marina*

Genetic structure and connectivity between populations of two common Mediterranean sessile invertebrates.

Tesi di laurea in Ecologia applicata

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II sessione

Anno Accademico 2011/2012

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Abstract

Population genetic and phylogeography of two common Mediterranean species were studied in 10 localities located on the coasts of Toscana, Puglia and Calabria. The aim of the study was to verify the extent of genetic breaks, in areas recognized as boundaries between Mediterranean biogeographic sectors. From about 100 sequences obtained from the mitochondrial Cytochrome Oxidase subunit I (COI) gene of *Halocynthia papillosa* and *Hexaplex trunculus* genetic diversity, genetic structure at small and large distances and demographic history of both specieswere analyzed. No evidences of genetic breaks were found for the two species in Toscana and Puglia. The genetic structure of *H. trunculus* evidences the extent of a barrier to gene flow localized in Calabria, which could be represented by the Siculo-Tunisian Strait and the Strait of Messina. The observed patterns showed similar level of gene flow at small distances in both species, although the two species have different larval ecology. These results suggest that other factors, such as currents, local dynamics and seasonal temperatures, influence the connectivity along the Italian peninsula. The geographic distribution of the haplotypes shows that *H. papillosa*could represent a single genetic pool in expansion, whereas *H. trunculus* has two distinct genetic pools in expansion. The demographic pattern of the two species suggests that Pleistocene sea level oscillations, in particular of the LGM, may have played a key role in shaping genetic structure of the two species. This knowledge provides basic information, useful for the definition of management plans, or for the design of a network of marine protected areas along the Italian peninsula.

1. Introduction

Recognizing biodiversity and biogeographical patterns, and how these relate to contemporary and past events is relevant to better predict the effects of future global environmental changes (Xavier & Van Soest 2012). In the last years, genetic diversity has been proved to be a crucial part of biodiversity and it's been demonstrated that can act as a good indicator of population fitness (Claudet et al., 2008). Gene flow may influence the genetic diversity by introducing new polymorphisms in a population, on which evolution can potentially act. This gain of variability into a population may also protect it from extinction (Demarchi et al., 2010; Pérez-Portela et al., 2010). Furthermore, the genetic structure of a species can indicate how related are populations and what is their level of connectivity. In the marine realm, connectivity was long time thought to have no restrictions. However, with the recent widespread use of molecular tools, has been seen that even in cases of well known and widely distributed species, patterns of connectivity are not so obvious, shaping intricate genetic networks and peculiar population structures (Schunter et al., 2011; Kim et al., 2012). For the majority of marine animals, it's possible to have a general idea of the dispersal capability of adults and larvae knowing the larval phase duration and their ability to move actively in the water column, or to be passively transported by currents and eddies (Bertness, 2001). However, direct observations on larval dispersal are scarce and difficult. Many works, in fact, infer the dispersal potential only by larval period measures on laboratory experiments and not with direct observations of larvae movements in the water column (Ayre et al., 1997).

An indirect way of studying connectivity is gene flow: it is known that the occurrence of an allele in populations distributed in geographic locations can be an evidence of gene flow between populations (Carvalho, 1998). Thus, gene flow can be inferred from spatial distribution of genetic markers by several statistical approaches (Kelly et al., 2010; Avise, 2004). The study of population connectivity using genetic tools has greatly increased in the last 30 years; nevertheless our understanding of these processes is still largely underdeveloped (Bertness, 2001). These patterns of connectivity, in fact, are related to a large number of physical factors and to specific physiological and ecological traits, which might affect different species in different ways (Toonen et al., 2011).

According with the physiological factors, gene flow between populations can be promoted principally by migration and dispersion of both larvae and adults (Bahri-Sfar et al., 2000). Species that have pelagic adults have the potential to move widely throughout the oceans and face few barriers to dispersal (Horne et al., 2008). Most marine benthic organisms have a biphasic life cycle with sedentary adults and dispersing gametes or larvae, which may be pelagic from few minutes to more than a year (Toonen et al., 2011). Planktotrophic larvae feed on particles and plankton found in the water column, and hence have the potential to move out of their point of release in the search of food (Durante & Sebens 1994). However, the majority of invertebrate larvae move into the water column like passive o semi-active particles (Barber et al., 2000). In fact, species with larvae that spend little time in the plankton should be rare at larger distances from their original habitat showing a low dispersal potential, whereas larvae spending longer periods in the water column should be able to enter on the principal marine currents, implementing their dispersal potential (Durante & Sebens 1994). An example of these last is the benthic stomatopod *Haptosquilla pulchella*, with a 4-6 week planktonic larval period, having a dispersal potential estimated around 600 km (Barber et al., 2000). On the other hand, some ascidian species, with a planktonic larval phase ranging from few minutes (colonial ascidians) to 24-36 hours (solitary ascidians), are observed only near to the point of larval release (Ayre et al., 2009; Lòpez-Legentil & Turon, 2006). However, it's important to note that some species with high larval dispersal have a strong population structure, whereas some others with a priori low larval dispersal capacity can show a little genetic structure and hence high gene flow between distant populations. These unexpected dispersal patterns are due to numerous physical, hydrological and historical factors, that may greatly shape connectivity patterns (Hart & Marko, 2010).

The most important physical factor that might modify connectivity in the marine realm is the currents regime, mainly defined by its direction and velocity. Other secondary factors can be listed as the extent of land masses and the salinity and isothermal gradients (Borrero-Pèrez et al., 2011; Jackson, 1986). The water current regimes at several scales, from the largest general circulation to the smallest local eddies, are capable to modify dramatically the connection between nearby areas. Big general circulations, as recorded for the sea cucumber *Holothuria mammata* across the Almerìa-Oran front, may negatively influence the connectivity pattern (Borrero-Pèrez et al., 2011). Not much information is available about the

larval duration of *H. mammata*, but it is considered to be a broadcast spawner, having a relatively long planktotrophic larval stage of 13–22 days as has been recorded for other Holothurian species.The restricted gene flow evidenced between the Atlantic and Mediterranean populations is presumably due to the Almerìan-Oran front, in the convergence of Atlantic and Mediterranean water masses (Borrero-Pèrez et al., 2011). Moreover, current regimes at smaller scales may affect the distribution of population as has been shown for the Indonesian mantis shrimps, whose connectivity between nearby populations (distance range of 1,500 km) is very low due to the strong currents crossing the Indian Ocean (Barber et al., 2000).

On the contrary, some species with hypothetical low dispersal capacity are homogeneous between distant populations, as described for the Mediterranean bath sponge *Spongia officinalis* (Dailianis et al., 2011). However the larval time stage is unknown for this species and, as for some others sponges, it has been hypothesized to be relatively short with the settlement phase close to parental substrata. In spite of this, the *S. officinalis* populations analysed at 11 locations along the Eastern Mediterranean, Western Mediterranean and the Strait of Gibraltar, appeared to be genetically differentiated between the three basins, but homogenous within each basin (Dailianis et al., 2011). The reduced genetic structure of *S. officinalis* inside Mediterranean sectors, although the inferred low dispersal capacity, may be due to the current regimes of the basins (Dailianis et al., 2011). At the same time, general currents can be modified in local scales by coastal morphology. Near the coast, the currents are modified in very unpredictable ways by the coastal morphology, and this represents the main problem when trying to predict gene flow in coastal or shallow water species (Bianchi, 2007).

The isothermal regime of the seas, both superficial and deep, is another important factor which may modify the connectivity pattern of the species. Many Mediterranean recognized barriers are represented just by the isotherms following annual seasons (Brasseur et al., 1996). The surface isotherms divide the Mediterranean Sea in sectors which can influence the distribution of species (Brasseur et al., 1996). As it will be shown later, the February 15 C° surface isotherm on the Strait of Sicily may represent a point of break for species distribution at both sides of the strait, while the February 14 C° surface isotherm appear to be related to the differentiation between the Ionian and the Adriatic Seas (Bianchi et al., 2007). The surface isotherms may also separate the distribution ranges of two closely related species with distinct temperature requirements, as is

the case of the gastropods *Charonia spp*, represented by *C. lampas lampas* in the western basin and by *C. tritonis variegata* in the Eastern basin (Russo et al., 1990). However, not only present day processes affect the connectivity between populations in the marine realm. Current biodiversity patterns reflect the interplay between both contemporary and historical processes, which can generate vicariance or differentiation processes by long time physical separation (Huntley & Birks 1983). The most important historical events causing successive vicariance processes are glaciations, during which the sea level decreased, followed by interglacial periods, during which the sea level raises again reconnecting neighboring basins (Carstens et al., 2009). After the reunification of the basins, the vicariance might have resulted in speciation (Boissin et al., 2011), but in some other cases this speciation process might not have been completed and populations can still interbreed, showing this events reflected in their DNA sequences (Stefanni & Thorley, 2003). This is the case, for instance, of the chaetognath *Sagitta setosa* in the Mediterranean Sea, which shows two distinct mitochondrial lineages, showing some historical vicariance event, but no full speciation, according to nuclear markers (Peijnenburg et al., 2006). All these historical events, together with life history traits, contribute to delineate the actual distribution and differentiation of species (Ayre et al., 2009).

Understanding which factors affect the current genetic structure of marine species and if this pattern is determined by geographic distance, currents, or by past history events, is among the most crucial question in current marine research (Hart & Marko, 2010).

1.1.Tools for the study of gene flow and connectivity

In the last 20 years, the study of DNA sequences has provided a powerful tool to investigate the relationships between extant populations, giving valuable information on its current gene flow by means of highly variable markers (microsatellite, nuclear DNA), as well as information on past history events when analyzing conserved markers (mtDNA, allozyme) (Templeton et al.,1995; Yoder & Yang, 2000).

Phylogeography is the field of molecular ecology that investigates the geographical distribution of genetic lineages within species and studies the factors that shape their observed genetic architecture (Yoder & Yang, 2000).

Phylogeographic studies are usually aimed to address large-scale evolutionary patterns, related to biogeographic barriers (Rocha et al., 2007). It can also study a group of species across the same distribution area, in order to identify congruent patterns of genetic lineage distributions and hence indicate areas conforming evolutionary significant units (Rocha et al., 2007). In this kind of studies, conserved sequences are necessary. Conserved DNA areas usually codify for vital proteins, and hence, have a slow mutation rate. Moreover, these kind of studies need DNA sequences with relative constant mutation rates, which is inversely proportional to DNA gene function. For that reason, conserved and constant sequences should be preferred for phylogeographical studies (Hellberg & Vacquier, 1999).

Population genetics focus on current connectivity patterns at large and small spatial scales (Kelly e Palumbi, 2010). Population genetics is based on direct observation of allele or haplotype geographic distribution and mutation, making use of allele/haplotype identity and the relationship among them to infer the level of gene flow between populations and their subdivisions at small spatial scales (Carvalho, 1998). A central problem in population genetics is detecting fine scale population subdivision, created by current gene flow (Kelly e Palumbi, 2010). In the case of population subdivision, the accuracy of the parameters might be small when based only on a few individuals or when the migration rate is small (Ayre et al., 1997). Moreover, if the study is not repeated in time, might be difficult to verify whether the pattern observed is restricted in space and time or general (Carvalho, 1998). In population genetics studies, in contrast to phylogeographyc studies, highly variable molecular markers are used. These markers should have a high mutation rate, and usually are found in non-coding regions, so they are useful to detect small-scale genetic differences in small, subdivided populations (Peijnenburg et al., 2006).

Different mitochondrial markers offer a good variability of mutation rate (Emerson et al., 2001). For example, in human mitochondrial DNA some parts which are not involved in coding, evolve faster than protein-coding regions (Emerson et al., 2001). The mitochondrial Cytochrome c Oxidase subunit I (COI) gene appears to be among the most conserved protein-coding genes in the mitochondrial genome (mtDNA) of animals. The mtDNA is transmitted predominantly through maternal lineages in most species and although physical recombination does occur; it is unusual or rare in many taxa (Avise, 2004). For those reasons, mtDNA genotypes are often referred to as molecular clones or haplotypes, and their inferred evolutionary interrelationships are interpreted as

estimates of "matriarchal phylogeny" (Avise, 2004). At the same time, the COI gene offers a good level of genetic variation in the third position of each codon, position that does not involve change in the aminoacid coded. This properties make the COI gene suitable for analyses at different spatial scales, from phylogenetic to population genetic analyses, as it can retain the signature of both past and present demographic events of populations and species (Pérez-Portela et al., 2010).

1.2.The Ecosystem based management

Understanding patterns of connectivity through gene flow has several practical applications such as, for instance, the identification of evolutionary units or the identification of different stocks of commercial species. It is therefore a powerful tool to make proper decisions about sustainable exploitation (Lòpez-Legentil & Turon, 2006). The implementation of conservational studies is receiving increasing attention, especially in the sea, given the recent emphasis in the Ecosystem-Based-Management (EBM) (Rocklin et al., 2011). The EBM can be defined as an integrated approach that considers the entire ecosystem, including its interactions as well as the cumulative impacts of all human activities (Botsford et al., 2009). To properly enforce EBM, it is mandatory to understand the patterns of distribution and connectivity of the species, from the largest to the smallest spatial scales, in order to recognize and preserve the relevant biodiversity units present in a specific habitat (Kalkan et al., 2011; Kelly et al., 2010). In that sense, it is known that a certain species distributed in many small isolated populations, is less capable to recover against any kind of natural or anthropogenic disturbance. This is true in terms of population size (number of individuals), as well as in terms of genetic diversity (Claudet et al., 2008). The genetic diversity at the population's level is given by the level of polymorphism and heterozygosity, which might work as a proxy of the health state or fitness of populations as biodiversity units (Botsford et al., 2009).

The most important application of an EBM is the correct implementation of Marine Protected Areas (MPAs). MPAs are especially protected zones that respond to a series of conservational plans assisted by management and economical rules (Perry et al., 2010). One of the first Mediterranean MPAs was created inside the Bonifacio Strait (Corsica) during the 1980s, responding to a decline of commercial species stocks and biodiversity (Rocklin et al., 2011). Since then, biological, ecological and genetics studies have shown that effective MPAs also serve to

promote both effective species protection as well as spill-over to non-protected areas, benefitting also fisheries (Rocha et al., 2007). Thus, the positioning of those areas is critical, and their success is maximized when guided by accurate information about the organism's dispersal potential, the patterns of connectivity among populations and the knowledge of biodiversity units (Bird et al., 2007). Molecular tools can give information not only on the connectivity between populations, but also can help to infer the optimal size of a protected area as has been seen in several instances (Rocklin et al., 2011). Molecular tools can detect the existence of different genetic pools, suggesting the most effective protection strategy, and suggesting for instance, the implementation of several linked MPAs, rather than a bigger one (Claudet et al. 2008).

1.3.The Mediterranean Sea

The Mediterranean Sea is a fully enclosed sea, except for a small connection with the Atlantic Ocean, the strait of Gibraltar, and recently with the subtropical Red Sea by the Canal of Suez. This basin is the largest (2,969,000 km^2) and deepest (average 1,460 m, maximum 5,267 m) enclosed sea, considered unique in the world for these features, but also for its peculiar hydrographic conditions, currents, temperature and salinity, which create a sharp gradient of conditions from West to East (Zulliger et al., 2009). In the Mediterranean Sea the evaporation is higher in its eastern half, causing the water level to decrease and salinity to increase from west to east. The resulting pressure gradient pushes cool, low-salinity water from the Atlantic across the Mediterranean Sea (Rizzo et al., 2009). The eastern region of the Mediterranean Sea is more oligotrophic than the western. Considering the whole Mediterranean basin the range of productivity decreases from north to south and west to east and is inversely related to the increase in temperature and salinity (Hamad et al., 2005).

The biodiversity of the Mediterranean Sea comprises approximately 17,000 species reported in the literature, among which at least 26% are prokaryotic and eukaryotic marine microbes. More than 8500 species are macro organisms, representing between 4% and 8% of the world's marine biodiversity (Coll et al., 2010). The main events structuring Mediterranean biodiversity pattern were the Atlantic flow of northern species during interglacial sea level rise, after 'Messinian Salinity Crisis' (MSC), and the invasion of Lessepsian subtropical species from the

Red sea after the recent opening of the Suez Canal (Boissin et al., 2011). Other recent climate change phenomena affect the oceanographic structure and biodiversity of the Mediterranean, among which tropicalization processes driven by global warming are the most evident (Bianchi & Morri, 2003).

All the physical, historical and environmental factors, together with recent genetic data along the basin, have permitted to define three main physical breaks which have been found to act as effective barriers to gene flow: the Almeria-Oran Front (AOF); the Bosphorous Strait, and the Strait of Sicily (Figure 1)(Quesada et al., 1995; Duran et al., 2004; Luttikhuizen et al., 2008).

The AOF is an oceanographic front exhibiting a pronounced step of temperature (1.4 °C) and salinity (2 ppt) gradient, over a distance of 2 km with an average water current speed of 40 cm/s. It flows south/eastward from the Spanish coast to the coast of North Africa (Tintore et al. 1988),and forms a genetic break for many sessile and pelagic species, as the mollusk *Mytilus galloprovincialis* (Quesada et al., 1995), the sea urchin *Paracentrotus lividus* (Duran et al., 2004), the chaetognat *Sagitta setosa* (Peijnenburg et al., 2006), as well as for many fish and algae species (Coll et al., 2010).

The Bosphorus Strait is a well defined barrier for many species, due to its particular characteristics: During the interglacial period of the Middle Pleistocene, the Mediterranean waters entered in to the Black Sea driving an invasion of Mediterranean fauna, which is believed to have destroyed most part of the Black Sea fauna (Nikula & Väinölä, 2003). Nowadays there is a mutual exchange of water among the two seas: the Mediterranean Sea flows through the Bosphorus Strait depleting itself of oxygen, whereas the Black Sea low-salinity waters flow in to the Aegean Sea, with similar characteristics, supporting the larval flow between the two basins (Nikula & Väinölä, 2003; Kalkan et al., 2011).

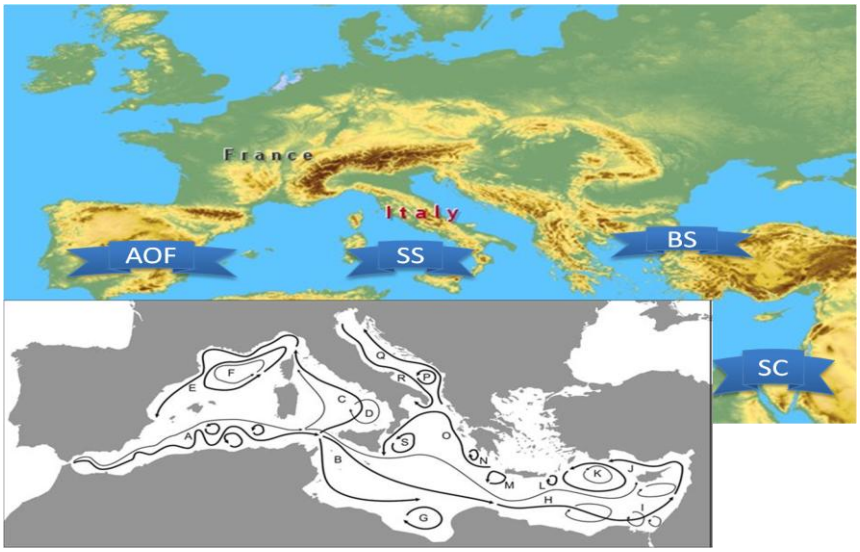

Figure 1: Geographical position of the main Mediterranean physical breaks that act as effective barriers to gene flow:AOF, Almerìa-Oran Front; SS, Strait of Sicily; SC, Suez Canal; BS, the Bosphorous Strait. In grey is shown the map of the main Mediterranean superficial fronts and currents (Bianchi, 2007).

In the central Mediterranean is located the Strait of Sicily, the area where the west and east side of the Mediterranean Sea are separated by a sharp reduction of depth and change in shallow currents (Stefanni & Thorley, 2003). In this area western currents flow on the clockwise direction, whereas Eastern currents flow on the contrary way, further complicated by small scale eddies and jets (Hamad et al., 2005). Moreover the Eastern basin is starved of phosphorous and is oligotrophic, enhancing the differences with the Western Mediterranean basin and strengthening this biogeographical break, described for many species (Krom et al., 2005). Many authors define the Strait of Sicily as the differentiation point of the paleo-biogeographical history of the Mediterranean Sea (Pancucci et al., 1999). In fact, the western basin shows a larger similarity with the Atlantic Ocean, hosting a higher number of cold-temperate species, while the eastern basin shows a large number of subtropical species (Bianchi & Morri, 2000). The Strait of Sicily represents a barrier to dispersal for many species, but also their meeting point. The presence, for instance, of the boreal-temperate species *Golfingia margaritacea* can indicate the prevalence, in that region, of colder water masses, whereas thermophilic species, such as *Phascolion convestium* and *Aspidosiphon elegans*, have been proposed as Lessepsian migrants (Tintore et al. 1988).

1.3.1.The central Mediterranean

It is interesting to note that most of Mediterranean fronts and basins are influenced by the biodiversity and connectivity of its central area, where the Italian peninsula is located. As already recognized by Astraldi et al., (1999) and Bianchi, (2007), the Italian coasts, as the Mediterranean Sea, seem to be divided in numerous sectors considering currents, isotherms and coastal morphology (e.g. land masses, bathymetric changes, islands) (Figures 2 and 3). The Tyrrhenian biodiversity is influenced by the western/central Mediterranean basin, where the Islands of Sardinia and Corsica represent the main geographical break points. The Sardinia channel (large about 1900 m) represents the main way for Atlantic-western Mediterranean flow into Tyrrhenian Sea (Astraldi et al., 1999). On the Tyrrhenian Sea it is recognized another break, splitting the southern and northern Tyrrhenian Sea (the Gulf of Lion and the Ligurian Sea) (Bianchi, 2007). This area, corresponding to the Toscana archipelago represents a break, where the larval flow might be modified as currents split in two different directions (Figure 3). Furthermore, the coastal morphology of the archipelago with its bathymetric oscillations can greatly modify larval dispersion (Fredj & Giaccone, 1995).

The Adriatic/Ionian subdivision is not so clearlydefined by shallow currents. The Ionian currents (counterclockwise) seem to not meet the western Mediterranean basin (clockwise) and the Adriatic ones (Figures 1 and 3). For most authors, the genetic differences detected in some species among the Adriatic and the others eastern basins are due not to a contemporary break, but rather to ancient migrations of the species from south eastern regions to northern western and north eastern regions (Stefanni & Thorley, 2003). The pattern of Adriatic Sea currents that flow through the Ionian Sea and other eastern basins are not well explained in literature, making difficult any description of whole Ionian Sea currents (Millot, 1992). Moreover, the February isotherm of the southeastern basin of the Mediterranean Sea changes inside the Ionian basin, dividing isotherm of Calabria coast by Puglia coast. This may represent a physical break (Figure 2) (Millot, 1992).

In the present thesis, taking into account the oceanographical and historical characteristics of the Mediterranean Sea, we identified three hypothetical barriers to gene flow located around the Italian peninsula: Toscana, corresponding with the southern/northern Tyrrhenian division, Puglia, corresponding with the Adriatic/Ionian division, and Calabria, corresponding with the Tyrrhenian/Ionian

division (Figure 3). This third area is particularly interesting since it has been suggested as the major boundary between Eastern and Western Mediterranean.

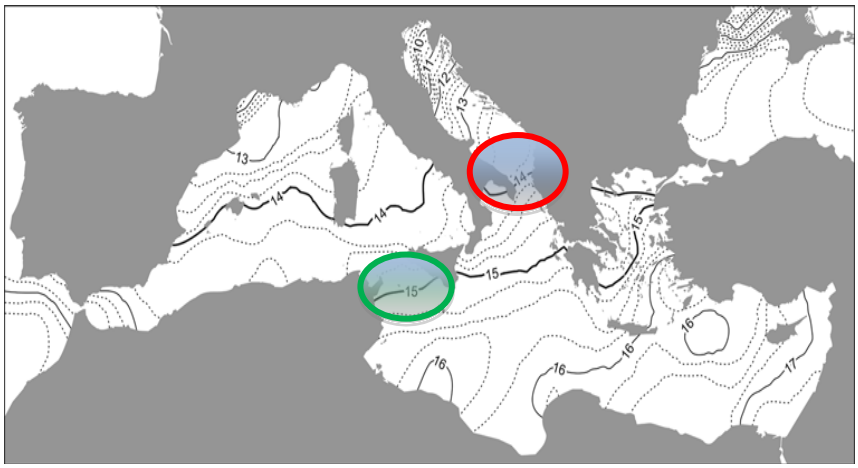

Figure 2: Surface isotherms of February of the Mediterranean Sea (climatological means from the historical data set 1906–1995). The 14 °C (red circle) and the 15°C (green circle) 'divides' are highlighted by a thicker tract. Modified after MEDATLAS (Brasseur et al., 1996).

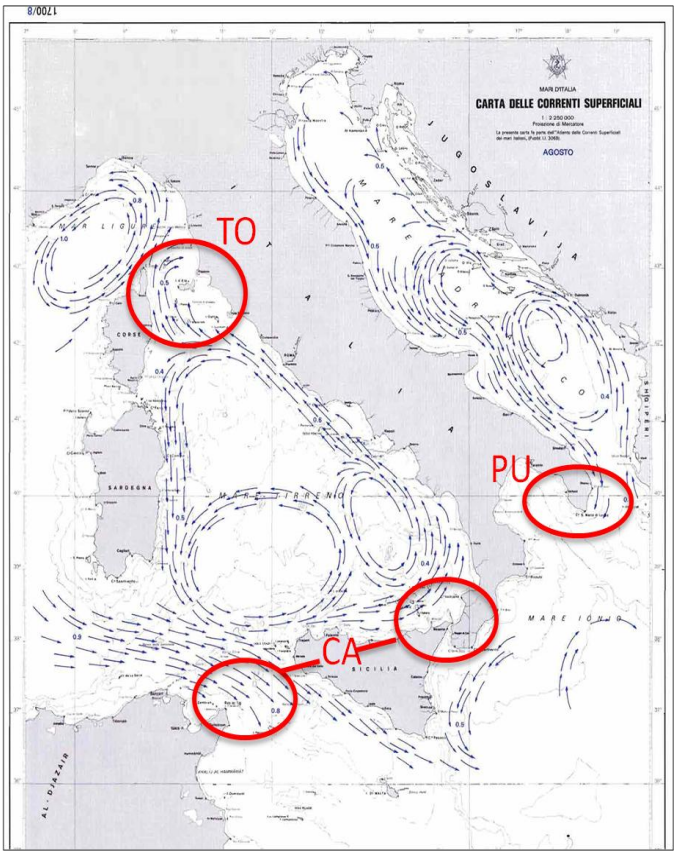

Figure 3: Map of superficial currents along the Italian coasts on August/September (Istituto Idrografico della Marina, Genova 1982). The red circles represent the location of the hypothetical break: TO, Toscana; CA, Calabria; PU, Puglia.

1.4.Thetarget species

The common assumption of past connectivity studies was that a single representative species could be used as a proxy to estimate dispersal among marine communities (Bird et al., 2007). During the last few years, it has been recognized that single-species studies on genetic connectivity were often contradictory considering the recognized barriers (Toonen et al., 2011; Bianchi 2007). Even among closely related species with similar ecology, life histories, and geographic ranges, the corresponding patterns of connectivity can be very different. In other cases species with highly divergent biology can have similar patterns of connectivity (Toonen et al., 2011). Such variability appears to be the rule rather than the exception, and has led to the necessity of multispecies comparisons.

Here, two widely distributed Mediterranean species living on rocky reefs, in the subtidal zone from 0 m to 200 m depth (Hofrichter, 2004; Bertness, 2001) were considered a two species study approach. The target species we have selected share some ecological characteristics as the geographic distribution, the bathimetric range and rocky substrata but they have some important differences as the reproduction seasonality and the adult mobility (*H. papillosa* is completely sessile where *H. trunculus* is able to make slow movements) (Šantić et al., 2010; Lahbib et al., 2010).

1.4.1.Halocynthia papillosa

Halocynthia papillosa (Figure 4) is an endemic Mediterranean ascidian of the order Stolidobranchiata, family Pyuridae. It is one of the most common solitary species on the rocky bottoms of the Mediterranean Sea (Šantić et al., 2010).*H. papillosa* is a non selective filter feeder, able to capture particles from 0.5 to 100 µm, with high retention efficiency for particles larger than 0.6 µm (Ribes et al., 1998).

Figure 4: *Halocynthia papillosa* on its habitat (http//www.commons. wikimedia.org).

Figure 5: oral pore of *H. papillosa*(http//www.commons. wikimedia.org).

As most sessile suspension feeder ascidians, these organisms are dependent on the surrounding water, which provides them with their resources (Ribes et al., 1998). Therefore, this species prefers the habitats exposed to intense water currents (Santic et al., 2010). Thus, the largest number of individuals of this species can be found on exposed habitats as protruding land masses in areas with high water flow and nutrient content (Ribes et al., 1998). This species is also considered a good indicator of water quality, due to its capacity of concentrating toxic elements in their tissues, such as heavy metals and hydrocarbons (Tarjuelo et al., 2001).

H. papillosa is a hermaphroditic species and broadcast spawner, with external fertilization that reproduces once per year in late summer. The chordate larvae are smaller than 500 µm of length and are lecithotrophic (Figure 6) (Šantić et al. 2010). The pelagic larval period of this species is not known, but for some others solitary ascidians it is thought between 12-24 hours, with a pre-competent period and prolonged metamorphosis of up to one week (Tarjuelo & Turon, 2004). *H. papillosa* is thought to have a relative high dispersal ability (Zeng et al., 2006). In fact, in a recent work Kim et al., (2012), studying the sister species *Halocynthia roretzi*, have shown that these species may have a larval period of up to two weeks. However, it has also been suggested that this larvae may use their swimming ability to stay close to the substrata of origin in search of appropriate settlement cues (Graham & Sebens, 1996).

Figure 6: Chordate Ascidiacean larva(http//etc.usf.edu.it).

1.4.2. Hexaplex trunculus

Hexaplex trunculus(also known as banded dye-murex, Figure 7) is one of the most common gastropods of the Mediterranean Sea and Atlantic bottoms. It is a medium-sized species of the family Muricidae, living principally on hard substrata but recorded also on soft bottoms (Zarai et al., 2011).

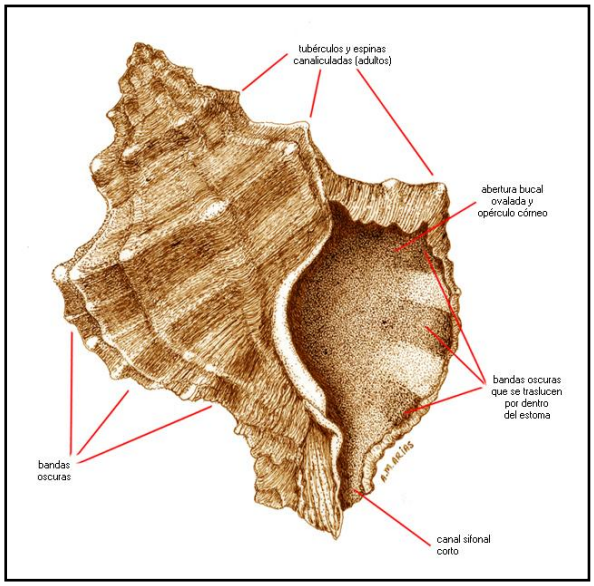

Figure 7: *Hexaplex trunculus* (http//www.commons. wikimedia.org).

While during the Roman Empire this species was exploited for its purple dye, nowadays it is also a commercially exploited species, mainly for human consumption, thanks to its nutritive properties, and its potential to replace some overharvested fishes and mollusc species (Zarai et al., 2011). For this reason, during the lasts years *H. trunculus* has been widely studied in order to describe its reproductive cycle, nutritive properties, and its response to pollution on coastal

habitats (Lahbib et al., 2010). In fact, some works evidenced that the shell and the body of *H. trunculus* can incorporate some pollutants suspended in the water column and this can constitute a problem for human consumption. Moreover, these pollutants also influence the body size and the reproductive cycle of this species, diminishing the number of propagules production (Abidli et al., 2012).

The banded murex *H. trunculus*, like most neogastropods, is gonochoric with internal fertilization (Fretter & Graham, 1994). The internal fertilization is performed following copulation and spawning of eggs within egg capsules. The incubation lasts 7 weeks, ending with the release of free juveniles (Figure 8). The time of gamete release is the same for male and female (from April to June) and seems to vary depending on habitat characteristics (currents and temperature oscillations) (Lahbib et al., 2010). Juveniles spend between 12 and 24 h searching for a suitable settlement surface (Lahbib et al., 2010).

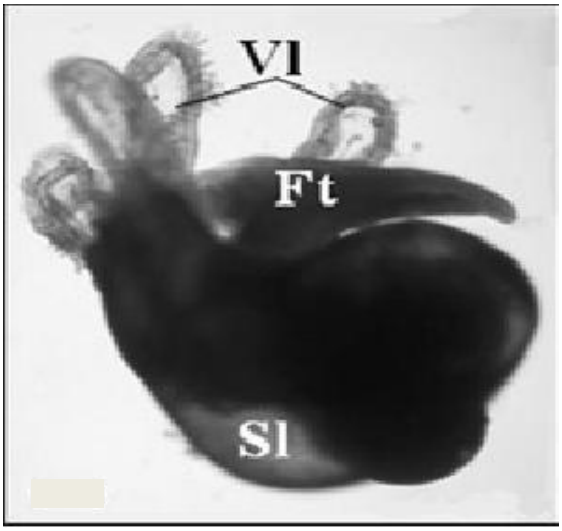

Figure 8:hatched veliger juvenile of *H. trunculus* (Lahbib et al., 2010).

2. Aim of this work

The purpose of the present thesis is to verify the presence of barriers to gene flow generating discontinuities in the distribution of genetic diversity in two shallow rocky benthonic invertebrates. This main goal can be subdivided in several specific objectives.

-To evaluate the effect of three potential barriers to gene flow located along the Italian coasts on connectivity patterns of two shallow rocky benthos species.

-As the two species studied *Halocynthia papillosa* and *Hexaplex trunculus* present contrasting reproductive modes and pelagic larval dispersal capacity, following the same sampling design for both species will allow to evaluate how life history traits can affect the population's genetic structure, its connectivity at several spatial scales, and how potential barriers to gene flow affects them.

-To understand large-scale genetic structure and connectivity around the Italian peninsula. Analyzing genetic differentiation among the three studied regions will give new information on phylogeographical patterns, and provide valuable data about genetic pools.

-By means of demographic studies we aim to understand how past history events affected the current genetic structure of the two species in the whole study area.

The number of species might be limited but it is useful to investigate the common connectivity patterns in the central Mediterranean Sea system along the Italian Peninsula. The importance, however, of past events genetic signal can be useful to improve our knowledge of genetic distributions during years and make inferences about resilience of the species and their response to future changes.

The results of this study, combined with the literature and with data collected in future studies, may also help to better implement an integrated and more comprehensive management and conservation plans, as for example the institution of marine protected areas (MPAs).

3.Materials and methods

3.1.Sample collection

Samples of *Halocynthia papillosa* and *Hexaplex trunculus* were collected between July 2011 and June 2012 by scuba diving from 5 to 20 m of depth from Tuscany, Puglia and Calabria (Figure 9). The sampling design was as it follows: in each of the three studied regions one population from each side of the hypothesized barrier were considered on the analyses, but for Toscana and Puglia at each side we sampled on two sites (Table 1). These two populations were about 100 km apart (Figure 9; Table 1). In each sampling site at least 30 individuals of each species were collected (Table 1). Whole individuals of both species were sampled, given that underwater dissection of a piece of tissue was difficult and, in the case of *H. papillosa*, did not avoid the death of the animal. Samples were immediately preserved in ethanol 80% and stored at +4 C° until processed.

Figure 9: Map of the sample sites: TOSnorth, Toscana north; TOSsouth, Toscana south; CALwest, Calabria west; CALeast, Calabria East; PUGwest, Puglia west; PUGeast, Puglia East. The red circles represent the location of the hypothetical break: TO, Toscana; CA, Calabria; PU, Puglia.(http//www.Google Chrome images.it).

Table 1: Regions, areas, sites, coordinates and number of individual collected of both species

Regions	***Populations***	***Sites of sample***	***Coordinates***	***Number of individuals collected***	
				H. papillosa	*H. trunculus*
Toscana	north	1 Torre di Calafuria	43°24'14.24"N/10°25'44.27"E	15	30
		2 Castiglioncello	43°36'20.28"N/10°21'14.07"E	30	15
	south	1 Talamone	42°33'19.73"N/11°07'59.87"E	30	30
		2 Porto Ercole	42°23'57.31"N/11°11'57.84"E	15	15
Puglia	west	1 Marina Serra	40°23'25.20"N/18°18'07.33"E	30	30
		2 Porto Badisco	40°08'52.90"N/18°29'08.71"E	16	15
	east	1 Rivabella	40°03'22.49"N/17°58'43.83"E	30	30
		2 Torre Inserraglio	40°15'44.72"N/17°53'51.17"E	15	15
Calabria	west	Vibo Marina	38°42'57.65"N/16°07'15.99"E	10	30
	east	Catanzaro	38°45'01.91"N/16°33'47.23"E	20	30
Tot.				211	240

3.2.DNA extraction and amplification

Total DNA was extracted using a REDExtract-N-Amp^{TM}Tissue PCR Kit protocol (SIGMA-ALDRICH). Total DNA was visualized in a 0,8% agarose gel, stained with Gelred (BIOTIUM) 1% after a 30 minutes electrophoresis at 120 V. The extraction product was diluted to 1:20 and 1:50 in ultrapure water (SIGMA) for better amplification success. A fragment of the mitochondrial COI gene was amplified with universal primers described in Folmer et al. (1994).As amplification was not consistent, from the first sequences obtained with the universal primers, specific primers were designed for each species with the software PRIMER vs.3.0 (http://www.fokker.wi.mit.edu/primer3/input.htm) (Table 2).

The PCR amplification reactions were performed in a 25 µL final volume consisting of: 2.5 µL of DNA template, 2.5 µL of buffer (PROMEGA), 2.5 µL of $MgCl_2$ 25 mM (PROMEGA), 2 µL of dNTPs 10 mM, 1.25 µL of each forward and reverse specific primers 10 mM (MACROGEN), 12.8 µL of ultrapure water (SIGMA) and one unit (0.2 µL) of Taq polymerase enzyme (Invitrogen**).** PCR reaction was performed in a GeneAmp® PCR Sistem 2700 thermocycler (Applied Biosytems). The amplification conditions were as follows: an initial denaturation at 94 °C for 3 min followed by 30/35 cycles of 94 °C for 45 sec, 45 sec at a specific annealing temperature (46/50 °C C for *H. trunculus* and *H papillosa* respectively) and an extension at 72 °C for 90 sec. After a final extension of 5 min at 72 °C products were maintained at 4ºC. Products of amplification were visualized in a 1,5% agarose gel stained as previously described.

Table 2: Table of universal primers and primers sequences for *H. papillosa* and *H. trunculus*.

Universal primers (Folmer et al., 1994)	_LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3'
	_HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'
Specific primer for *Halocynthiapapillosa*	_F 5'-TTG TTT GGT GTT TGG TCT GG-3'
	_R 5'-GCA GCT GCC AAT ACT GGT AAA-3'
Specific primer for *Hexaplex trunculus*	_F 5'-ACG GCC GTT TTA CTC CTT CT-3'
	_R 5'-ACC GGT TAT TTT CCC GAA TC-3'

PCR products were sent to Macrogen Europe Inc. for purification and sequencing. Sequences were checked by eye and edited with ChromasPro vs1.5 (www.Technelysium.com.au/ChromasPro.htlm). Alignments were performed using MEGA 5 (Tamura et al 2011).

3.3.Data analysis

3.3.1.Genetic diversity of populations

Nucleotide diversity π, number of haplotypes *h*, haplotype diversity *Hd,* number (N_p) and the percentage of private haplotypes ($\%N_p$) were calculated using DnaSP v5.10 software (Rozas et al., 2003) for all populations at each side of the hypothetical barriers and for the three regions (Table 1). The number of private haplotypes gives a further indication of the diversity of a population (Kelly et al., 2010).

The relationships between haplotypes were represented in a haplotype network, performed with the software Network v4.6.1.0 (Fluxus Technology Ltd.).The Median-Joining network is implemented showing the differences between aligned sequences of one or more populations in a network of distance measures. The simplest way to obtain a distance measure between two sequences is to count the number of character differences and weight the character changes (Bandelt et al., 1999).It is based on improved Kruskal's (1956) algorithm, which lists triplets of sequences in increasing order of distance. The resulting network shows the sequences or individuals grouped together according to the haplotypes recognized and their relative distances (Bandelt et al., 1999*)*. This type of network allows identifying haplotypes that are more frequent among a group of individuals, a location, populations that share haplotypes or not etc. The haplotype network gives, together with diversity index (haplotype diversity, number of private haplotype), a

good representation of population genetic structure and some indirect indications of actual population size (Hart & Marko, 2010).

3.3.2.Population differentiation

To study similarity between populations the F_{st} index between each pair of populations was calculated. F_{st} can be interpreted as the proportion of genetic variation distributed among and within subdivided populations and indirectly of the level of gene flow between populations (Beerli & Felsenstein 1999). F_{st} values range between 0, indicating that populations are genetically identical, and 1, indicating that populations are differentiated according to the specific alleles. To better visualize the results of pairwise F_{st} values, we represented on a bidimensional space the matrix of dissimilarity in a Multidimensional scaling representation (MDS), using the Primer software (Clarke & Ainsworth, 1993).

According to the results of pairwise F_{st}, we grouped populations in homogeneous groups, where no significant genetic differentiation was detected. We analyzed the Molecular Variance of these groups, AMOVA, implemented in Arlequin software v3.5.1.2 (Excoffier & Lischer 2010). The Analysis of molecular variance assigns percentages of variability explained and a significance to the variability among groups, within populations inside the groups and within populations without grouping, giving information on the degree of homogeneity of the groups set and how differentiated are from each other.

Finally, the Isolation by distance model was tested with a Mantel test (Mantel, 1967) with 1000 permutation in Arlequin v3.5.1.2 (Excoffier & Lischer 2010). The Mantel test calculates the correlation between two matrices, one of them is the genetic distance as $F_{st}/1\text{-}F_{st}$ and the other is the matrix of log-transformed geographical distances between populations. The isolation by distance model predicts that genetic differentiation will be proportional to geographic distance. If Mantel test results not significant, no correlation between the two matrices exists, and hence it should be inferred that some factors other than distance are affecting the differentiation between populations.

Finally, with the software MIGRATE (Beerli & Felsenstein 1999), we inferred the approximate number of migrants between these three regions. MIGRATE uses a method to make a maximum-likelihood estimate of population parameters for

geographically subdivided populations, using gene frequency distribution and including on this method the Coalescent theory (Beerli & Felsenstein 1999).

3.3.3.Demographic analysis

Demographic analyses allow to evaluate the changes on population size through time and infer information on the behavior of populations during past history events (Rogers & Harpending, 1992). It is known that changes in population size left signatures in DNA sequences as proportion of differences, so mismatch distribution analysis are calculated as the frequency of pairwise differences between haplotypes, which can be represented in a graph for better interpretation. The mismatch distribution method is based on the assumption that a rapid population expansion is plotted on the line graph like a peak, as there is a high frequency of individuals with small number of pairwise differences, whereas stable population size has a multimodal distribution of growths and declines (Figure 10) (Harpending, 1994). Mismatch distribution also allow the detection of subpopulations, different genetic pools that coexist in the same geographic area. In figure 10 are shown the most common types of mismatch distribution that populations can show.

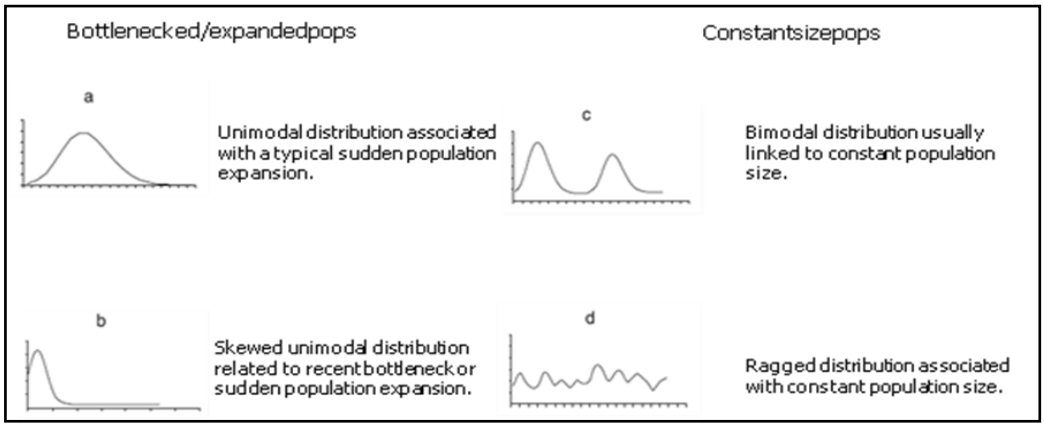

Figure 10: Main types of mismatch distributions: (a) unimodal distribution associated with a typical sudden population expansion; (b) skewed unimodal distribution related to recent bottleneck or sudden population expansion; (c) bimodal distribution usually linked to the presence of more than one genetic pool; (d) ragged distribution associated with constant population size (Patarnello et al.,2007).

For *H. papillosa* the mismatch distributions for the whole sequences and for the three regions were calculated, while for *H. trunculus* the mismatch distributions of the two groups found on F_{st} analyses.

After, two different tests of selective neutrality, Tajima's *D* (Tajima 1989) and Fu's *Fs* (Fu 1997) were performed. The classic neutral theory was described

initially for ecologically studies of populations' connectivity (Kimura, 1968). However, the neutrality theory applied to molecular ecology states that "mutation substitutions on sequences are caused by random changes of selectively neutral marker under continued mutation pressure" (Kimura, 1991). Alleles at a given locus are selectively neutral, i.e. substituting one allele by another does not affect the fitness of an individual or of a population (Rosindell et al., 2011). Regarding a neutral marker, at large time scales it is notable that mutations would not affect in a large extent the resilience of populations in terms of genetic complexity. The molecular variability can be hence described as a function of neutral mutation rate and effective population size (Avise, 2004).

The Tajima's D relates the number of segregating sites and the average number of nucleotide differences estimated with pairwise sequences comparison (Tajima, 1989), while Fu's test compares the number of slightly different alleles (typical of expanding populations) with the number of alleles statistically expected in a expanding population (Fu, 1997). Significant negative *D* and *Fs* values mean significant departures from mutation-drift equilibrium of populations and can be interpreted as signatures of population expansion. In the opposite, positive values can be interpreted as situations of constant size and in equilibrium populations (Patarnello et al., 2007).

The approximate time of expansion was calculated for those populations fitting the sudden expansion model with the Harpending (1992) equation:

$$T=2\hat{u}t;\ \hat{u}=2\mu k$$

Where *t* is the time expressed in generations and $\hat{u}$ is the per-generation probability of a sequence to have a mutation anywhere. The term û is given by the average number of nucleotide *k* of the COI amplified sequences and by the term μ, the mutation rate per nucleotide which changes according to different species. Although genes mutate at different rates according to their characteristics (mtDNA, nuclear DNA, allozymes), the common assumption that genes evolve with a nearly constant rate at long time scales is accepted (Hellberg & Vacquier, 1999). For an expanding population, knowing the mutation rate per nucleotide and the number of nucleotides of a sequence, is possible to calculate the time when the expansion began and relate it to a particular historical event (Rogers & Harpending, 1992). The general mutation rates applied for gastropods'COI is 2.4% per million years

(Hellborg & Vacquier 1999). The mtDNA mutation rate of *H. papillosa* is not reported on literature but that of*H. roretzi* is estimated in 2.86% per million years so it is possible that *H. papillosa* has the same (Kim et al., 2012).

4.Results

4.1.Halocynthia papillosa

4.1.1.Genetic diversity

A portion of 371bp of the COI gene was obtained from 113 individuals of *Halocynthia papillosa*: 58 individuals from Toscana, 40 from Puglia, and 14 individuals from Calabria (Table 3). A total of 19 haplotypes were obtained, where 9 of them were private, 7 of which were found in Toscana populations (Table 3).

The haplotype diversity (*Hd*) and nucleotide diversity (π) detected for the whole data set of this species are, respectively, 0.853 and 0.006, similar to those found for each region independently (Table 3).

Table 3: Table of diversity index of *H. papillosa:N*, number of sequences; *h*, number of haplotypes; *Hd*, haplotype diversity± standard deviation; π, nucleotide diversity± standard deviation; *Np*, number of private haplotypes; *Np%*, percentage of private haplotypes. Code: TOSnorth, Toscana north; TOSsouth, Toscana south; CALwest, Calabria west; CALeast, Calabria east; PUGwest, Puglia west; PUGeast, Puglia east.

Regions	*Code*	*Halocynthia papillosa*					
		N	*h*	*Hd*	π	*Np*	*Np%*
Calabria	*CALwest*	7	3	0.714(±0.016)	0.007(±0.000)	1	33.3
	CALeast	7	6	0.952(±0.009)	0.008(±0.000)	0	0
Puglia	*PUGwest*	25	5	0.777(±0.005)	0.003(±0.000)	1	20
	PUGeast	15	5	0.790(±0.001)	0.004(±0.000)	0	0
Toscana	*TOSnorth*	29	11	0.872(±0.001)	0.006(±0.000)	4	36.4
	TOSsouth	29	8	0.837(±0.001)	0.005(±0.000)	3	27.3
Total		113	19	0.853(±0.000)	0.006(±0.000)	9	47.4

In the haplotype network, it is possible to see that the two more frequent haplotypes (H_7 and H_8) are common in Toscana populations which also present large number of low frequency and private haplotypes, closely linked to H_7 and H_8 (Figure 11). As already evidenced on Table 3, Toscana has more private haplotypes than Puglia (only one, H_11). The H_1 is more common in Calabria, which also shows other common haplotypes, as H_7 and H_3, less common as the H_4, H_5, H_2, and one single private haplotype, H_6 (Figure 11).

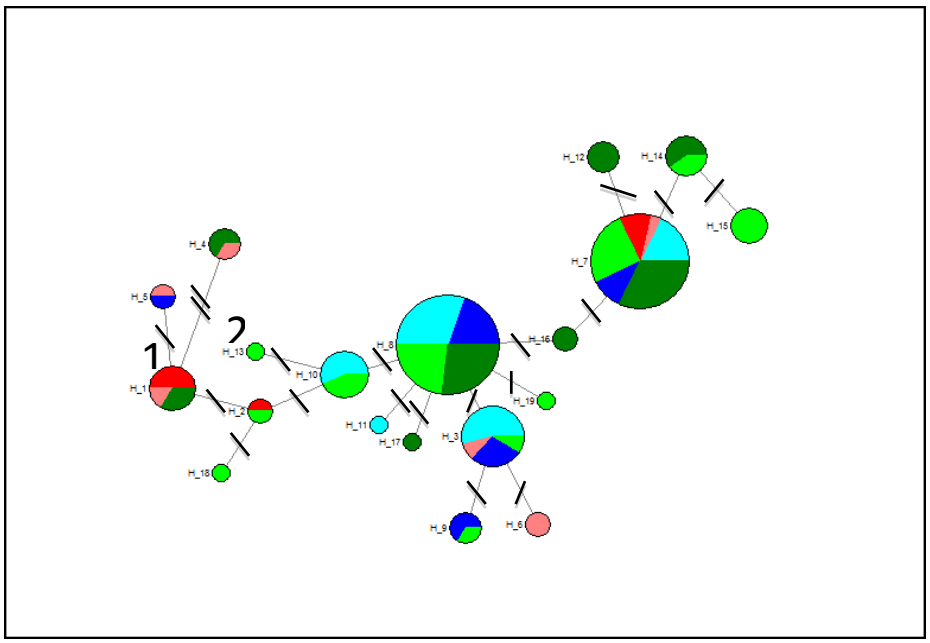

Figure 11: Haplotype network of *Halocynthia papillosa*. Colors represent the populations: TOSnorth, light green; TOSsouth, dark green; CALwest, red; CALeast, pink; PUGwest, blue; PUGeast, light blue. Short black lines represent the number of mutations between the haplotypes. Two black lines with number 2 represent two mutation where one black line represent one mutation. The number 1 represents one mutation and the 2 number represents two mutations.

4.1.2.Population differentiation

The pairwise F_{st} analyses do not show significant differentiation between populations located at each side of the hypothesized barriers in any of the three regions (Table 4). Between regions, F_{st} values show small but significant differences in all pairwise comparisons, except between TOSsouth (the south area of Toscana) and CALwest (the west area of Calabria) (Table 4). When pooling together populations of each region all three regions show significant differences between them, however Puglia seems to be more similar (in terms of F_{st} value) to Toscana than Calabria (Table 5). The highest differences occur between Calabria and Toscana, with a differentiation value of 0.378 and P=0.000 (Table 5).

The pairwise differences between all the populations are represented in the multidimensional scaling (Figure 13), where populations belonging to the same region are closer together.

Table 4: Table of pairwise differences (F_{st}) between *Halocynthia papillosa* populations: TOSnorth, Toscana north; TOSsouth, Toscana south; CALwest, Calabria west; CALeast, Calabria east; PUGwest, Puglia west; PUGeast, Puglia east.

	CALwest	*CALeast*	*PUGwest*	*PUGeast*	*TOSnorth*	*TOSsouth*
CALwest	0.000					
CALeast	-0.006	0.000				
PUGwest	**0.210***	**0.158***	0.000			
PUGeast	**0.278****	**0.284*****	-0.033	0.000		
TOSnorth	**0.105***	**0.223*****	**0.087***	**0.087***	0.000	
TOSsouth	0.098	**0.252*****	**0.170*****	**0.171*****	0.015	0.000

* = P< 0.05; **=P< 0.01; ***= P<0.000.

Table 5: Table of pairwise differences (F_{st}) of *Halocynthia papillosa* regions when population within regions are pooled together.

	Calabria	*Puglia*	*Toscana*
Calabria	0.000		
Puglia	**0.341*****	0.000	
Toscana	**0.378*****	**0.103*****	0.000

*=P< 0.05;**=P< 0.01; ***= P<0.000.

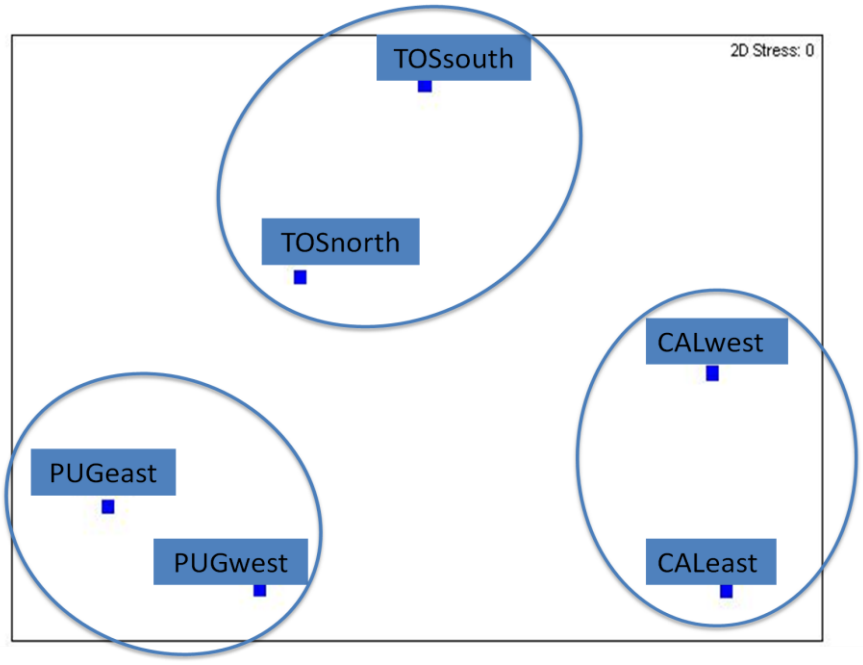

Figure 12: Multidimensional scaling (MDS) of the 6 populations of *H. papillosa*. Blue circles include the groups evidenced by F_{st} analyses.

According to the F_{st}values obtained, we run the AMOVA analyses with three groups corresponding to the three regions (Table 6). The results show that most of the variability is due to differences within populations (94.72%, P=0.005 whereas the variability among groups explain only the 4.99% of the total variation (P=0.078).

Table 6: Table of AMOVA of all populations of *H. papillosa*.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P value
Among groups	2	2.309	0.021	4.99	0.078
Among populations within groups	3	1.307	0.001	0.29	0.394
Within populations	107	44.127	0.412	94.72	0.005
Total	112	47.743	0.435		

Significance when P< 0.05

For *H. papillosa* the Mantel test did not detected a significant correlation between pairwise differences among populations and geographic distance (P=0.16), indicating that the genetic differentiation detected between regions is not proportional to geographic distance, as expected following pairwise F_{st} values.

The MIGRATE results show a great effective population size on Toscana, with an average Θ value of 0.016. The most relevant migration rate seems to be from Calabria to Puglia (mean=629.7) whereas the opposite migration rates from Toscana and Puglia to Calabria are much lower (Figure 13-14). The migration rate from Calabria to Toscana is not symmetric and with a higher variance. Between Puglia and Toscana we also detect a not perfectly symmetric migration but relevant, with a mean of 604.3(Figure 13-14).

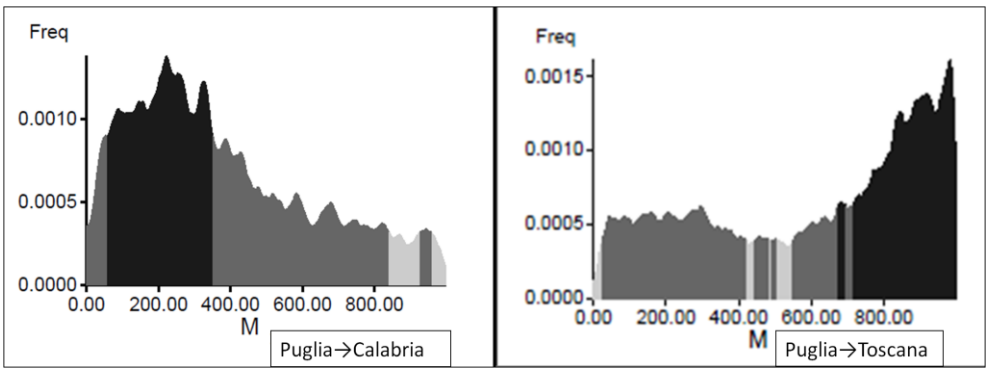

Figure 13a: *H. papillosa* migration rates of Bayesian Analyses Posterior Distributions. The black area indicates the highest probabilities.

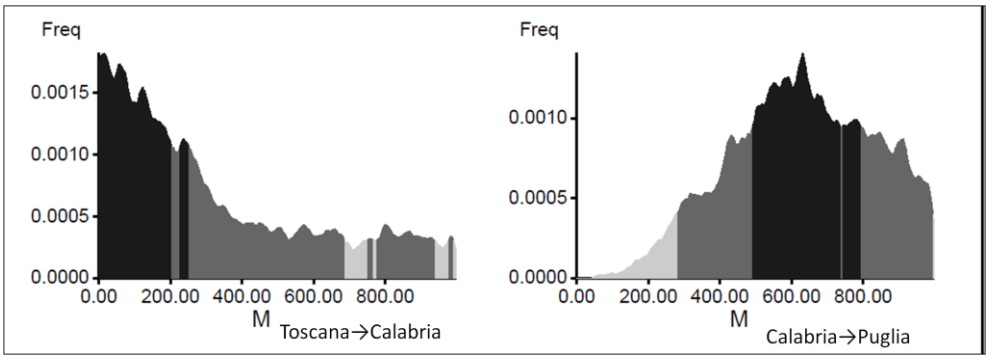

Figure 13b: *H. papillosa* migration rates of Bayesian Analyses Posterior Distributions. The black area indicates the highest probabilities.

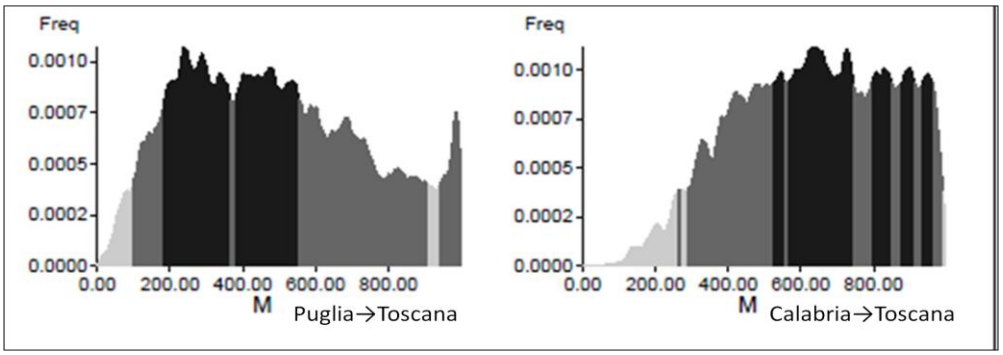

Figure14:*H. papillosa*migration rates of Bayesian Analyses Posterior Distributions.

4.1.3.Demographic analysis

The mismatch distribution of the whole data set of *Halocynthia papillosa* fits to a pattern of sudden expansion (goodness of fit=0.001P=0.770) (Figure 15). Because no significant differences were detected inside each region (Table 4), we only performed the mismatch distributions of each region separately (Figures 16-17-18).

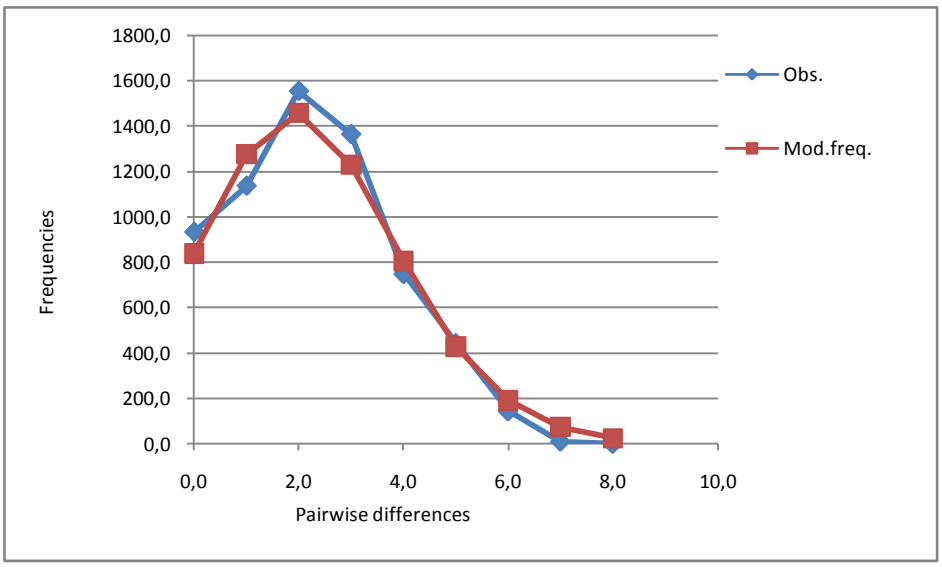

Figure 15: Mismatch distribution of the whole data set of *Halocynthia papillosa*.

For the whole data set, Tajima's D test was positive and not significant (D=1.224; P=0.908), while Fu's was negative and highly significant (F=-17.458 P=0.000) (Table 7), indicating population expansion. The mismatch distributions of the regions fit to a pattern of sudden expansion model, with significant Fu's neutrality test (Figure 16-17-18 and Table 7). Tajima's D neutrality tests were not significant in any case (Table 7), but Fu's test has been suggested to be more powerful in detecting neutrality (Avise, 2004). As calculated with Harpending, (1992) equation, *Halocynthia papillosa* experienced a recent expansion, about 17700 years ago (μ=2.86/million years; 1 generation/year).

Table 7: Table of mismatch goodness of fit and neutrality tests of the three regions. Significant values when P<0.05.

Regions	*Goodness of Fit*	*Tajima's D*	*Fu's Fs*
Calabria	0.003(P=0.950)	0.171(P=0.591)	-4.038(P=0.009)
Puglia	0.001(P=0.690)	-1.137(P=0.118)	-6.632(P=0.001)
Toscana	0.000(P=0.940)	-0.832(P=0.234)	-17.458(P=0.000)
All seq.	0.001(P=0.770)	1.224(P=0.908)	-7.100(P=0.010)

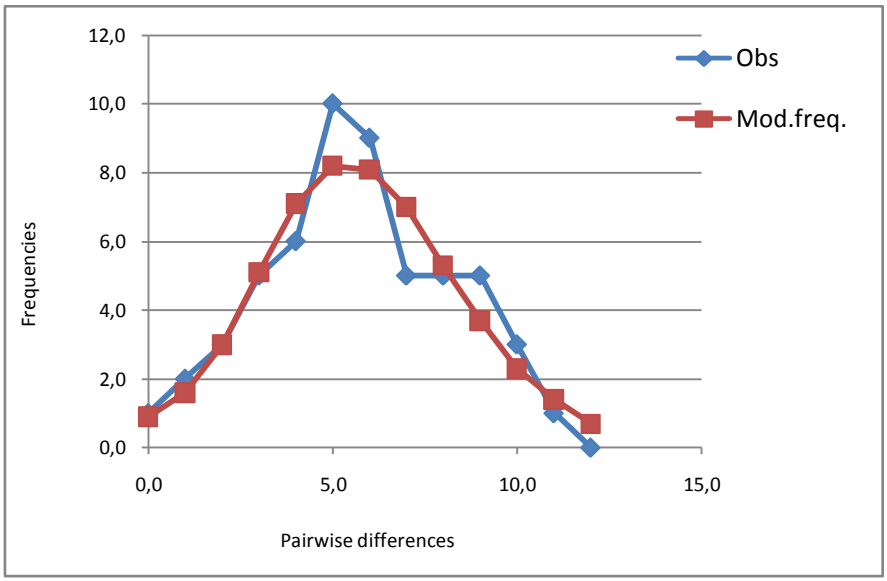

Figure 16: Calabria mismatch distribution of *H. papillosa*.

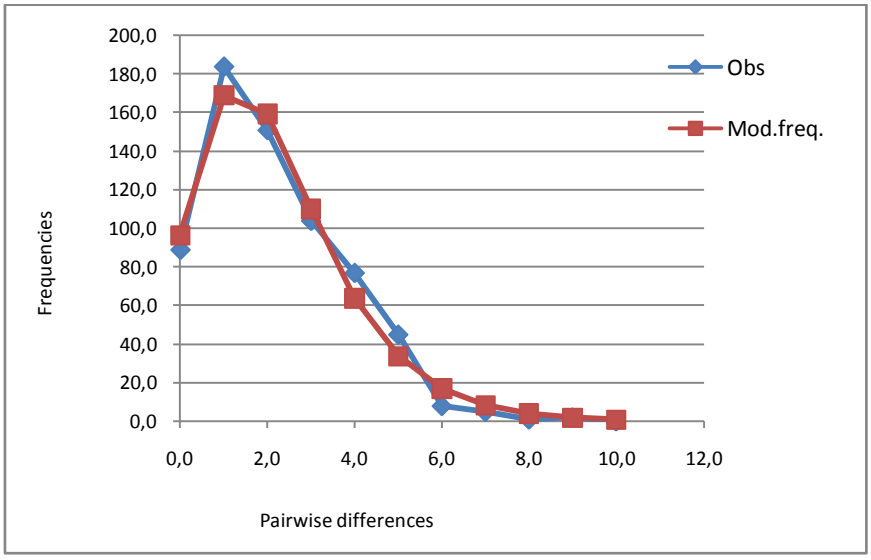

Figure 17: Puglia mismatch distribution of *H. papillosa*.

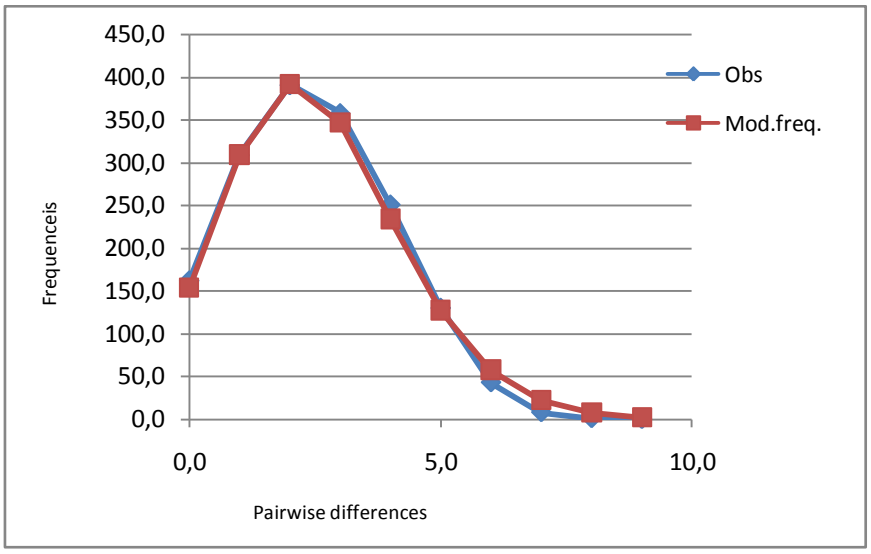

Figure 18: Toscana mismatch distribution of *H. papillosa*.

4.2.Hexaplex trunculus

4.2.1.Genetic diversity

Sequences of 523bp of the mitochondrial gene COI from 100 individuals of *Hexaplex trunculus*were obtained. The number of sequences (*N*) obtained by region was more homogenous than in the case of *H. papillosa*: 36 sequences from Toscana, 37 from Puglia and 27 sequences from Calabria (Table 8).

Out of 27 haplotypes (*h*)9 of them were found only in Calabria, 8 in Puglia, and 5 in Toscana (Table 8). The haplotype diversity (*Hd*) of the whole data set is slightly lower than that seen in *H. papillosa* (0.753), and the same is true for each of the three regions (Table 8). The smallest values of *Hd*and π are found in Toscana, whereas the highest values are found in Calabria (Table 8).

Table 8: Table of genetic diversity index of *H. trunculus*:*N*, number of sequences; *h*, number of haplotypes; *Hd*, haplotype diversity± standard deviation;*π*, nucleotide diversity± standard deviation; *Np*, number of private haplotypes; *Np%*, percentage of private haplotypes. Populations: TOSnorth, Toscana north; TOSsouth, Toscana south; CALwest, Calabria west; CALeast, Calabria east; PUGwest, Puglia western; PUGeast, Puglia east.

Regions	*Code*	*Hexaplex trunculus*					
		N	*h*	*Hd*	*π*	*Np*	*Np%*
Calabria	*CALwest*	12	5	0.803(±0.006)	0.006(±0.000)	2	40
	CALeast	15	9	0.848(±0.007)	0.012(±0.000)	7	77.7
Puglia	*PUwest*	17	8	0.846(±0.003)	0.013(±0.000)	5	62.5
	PUeast	20	6	0.763(±0.004)	0.011(±0.000)	3	50
Toscana	*TOSnorth*	24	5	0.312(±0.010)	0.003(±0.000)	4	80
	TOSsouth	12	2	0.167(±0.010)	0.000(±0.000)	1	50
Tot		100	27	0.753(±0.001)	0.013(±0.000)	22	81.5

The haplotype network of *Hexaplex trunculus*shows two well distinct clades separated by 10 mutations, each one of them showing a clear star-like shape (Figure 20).The clade-A is characterized by one more common haplotype (H_3), one less common haplotype (H_7) and few other private haplotypes (Figure 19). The haplotypes of clade-A, are present only in Puglia (light and dark blue), Calabria east (pink) and in a single individual (H_27) of Calabria west (red). The clade-B seems to be more polymorphic and geographically widespread, with haplotypes present in all the populationsand in the three regions. The H_4 is the most common haplotype on the clade B and appears in Puglia, Calabria and Toscana (Figure 20). There is also an individual of Toscana north (H_16) linked with more than 18 mutation steps to haplotypes of Calabria individuals (Figure 19).

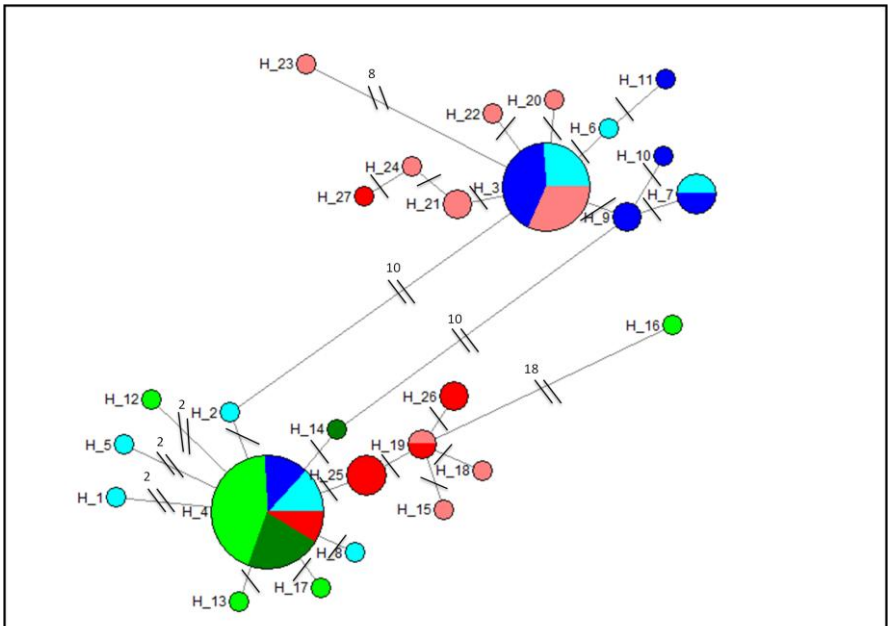

Figure 19: Haplotype network of *Hexaplex trunculus*.Colours represent the populations: TOSnorth, light green; TOSsouth, dark green; CALwest, red; CALeast, pink; PUGwest, blue; PUGeast, light blue. More than one mutation is signed with two black lines and numbers; one line is one mutation. Number 2 indicates two mutations, number 8 indicates eight mutations, number 10 indicates ten mutations and number 18 indicates eighteen mutations. The upper right clade is clade-A, the lower left clade is clade-B.

4.2.2.Population differentiation

The F_{st} values show no pairwise differences among the populations inside Puglia and Toscana (Table 8). Inside Calabria, a sharp differentiation between the two populations was detected (F_{st}=0.531, P=0.000).Moreover, Toscana and Puglia are significantly different (Table 8). The western population of Calabria (CALwest) shows small but significant differences with Toscana and larger differences with Puglia. The eastern Calabria population (CALeast) shows small differences with Puglia populations (Table 8).

The representation of these F_{st} values shows how populations inside Puglia and Toscana are grouped together, whereas the two regions are highly distinct (Figure 20). It is also represented the large differentiation between the two populations of Calabria (Figure 20). It is also possible to recognize two clear groups that fit F_{st} pairwise differences: one group, (Southern Group hereafter), formed by Puglia and Calabria east populations, and a second group (Northern Group hereafter) including Toscana and Calabria west populations.

Table 8: Table of pairwise differences of all populations of *Hexaplex trunculus*: TOSnorth, Toscana north area; TOSsouth, Toscana south area; CALwest, Calabria west area; CALeast, Calabria east area; PUGwest, Puglia west area; PUGeast, Puglia east area.

	CALwest	*CALeast*	*PUGwest*	*PUGeast*	*TOSnorth*	*TOSsouth*
CALwest	0.000					
CALeast	**0.531*** **	0.000				
PUGwest	**0.480*** **	0.026	0.000			
PUGeast	**0.263*** **	**0.145***	0.032	0.000		
TOSnorth	**0.160*** **	**0.670*** **	**0.592*** **	**0.376*** **	0.000	
TOSsouth	**0.222*** **	**0.664*** **	**0.581*** **	**0.347****	-0.028	0.000

Signification of * at P< 0.05;**, P< 0.01; ***, highly significant at P=0.000.

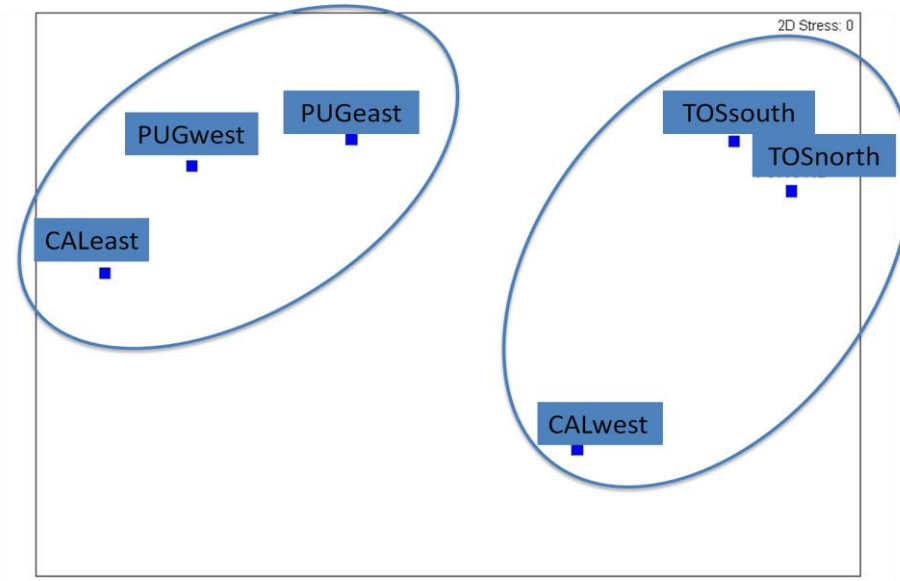

Figure 20: MDS of pairwise F_{st} values between all populations of *H. trunculus*.The Northern Group includes down right side populations TOSnorth, TOSsouth and CALwest, and the Southern Group include upper left side populations, PUGwest, PUGeast and CALeast. Blue circles include the two groups found on F_{st} analyses.

The AMOVA analyses were conducted for the two groups detected according to F_{st} values, Southern Group and Northern Group. The highest variation is found within populations (68.95%) and among groups (17.41%) however this last is not significant (Table 9). The F_{st}between the Southern Group and Northern Group is small (0.257) but highly significant (Fst=0.257; P=0.000)

Table 9: Table of AMOVA of the two groups of *H. trunculus*. The Northern Group is compost of Toscana and Calabria west populations; the Southern Group is compost of Puglia and Calabria east populations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P value
Among groups	1	5.577	0.073	17.41	0.341
Among populations within groups	2	2.719	0.057	13.64	0.000
Within populations	93	27.106	0.291	68.95	0.000
Total	96	35.402	0.422		

Significationat P< 0.05.

The low P value shown among groups (P=0,341), although the percentage of variation is evident (17,41%), is probably due to the fact that these two groups (Figure 21) do not correspond to the real segregation of individuals inside the two clades recognized on the haplotype network (Figure 19). For this we separated the individuals of Puglia and Calabria east belonging to the two clades and performed the AMOVA between the two clades (Table 10). In this case we did detect a significant percentage of variation explained among these two clades (42.14%, P=0.010). F_{st} value between clade-A and -B is highly significant (F_{st}=0.451; P=0.000).

Table 10 AMOVA betweenthe two clades of *H. trunculus*. Clade-A and clade-B are described above and are shown on haplotype network (Figure 19).

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P value
Among groups	1	9.623	0.20455 Va	42.14	0.010
Among populations within groups	7	4.388	0.03780 Vb	7.79	0.000
Within populations	88	21.390	0.24307 Vc	50.07	0.000
Total	96	35.402	0.48542		

Signification at P< 0.05.

The Mantel test not showed significant correlation between the genetic diversity and geographic distance (P=0.05).

MIGRATE analyses indicate that Southern Group shows a mean effective population size of Θ=0.022, much higher than that of the Northern Group (Θ=0.004). The migration rate between the two groups is asymmetric: the migration

from the Northern Group to the Southern Group is higher than in the opposite direction (mean values of 692.5 and 284, respectively; Figure 21).

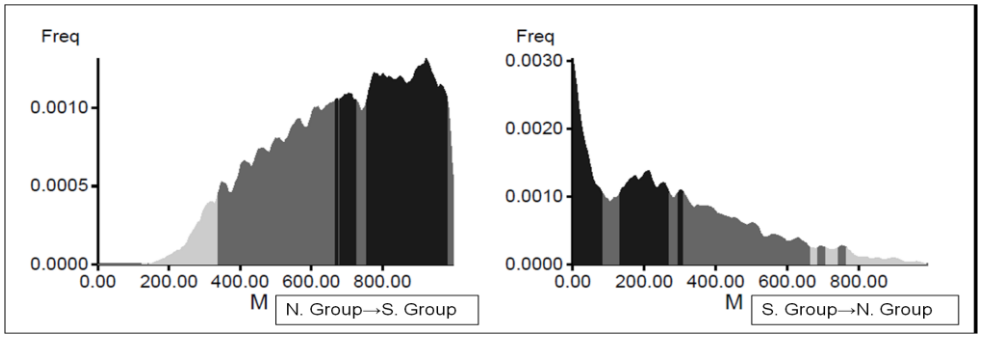

Figure 21: Migration rates Bayesian Posterior Distributions. S. Group=Southern Group; N. Group=Northern Group.

4.2.3.Demographic analyses

The mismatch distributions and neutrality tests were calculated for the two groups according to pairwise F_{st}values (Table 9). We did the same for the two clades identified in the haplotype network (figures not shown).

The Northern Group mismatch distribution shows a typical form of population expansion, with a peak corresponding to high frequencies of low pairwise differences (Figure 23). The plot fits to a sudden expansion model, and the neutrality tests are both negative and significant (Table 11).

Table 11: Goodness of fit to the sudden expansion model, and neutrality tests of the two groups and two clades.

Group/lineage	*Populations included*	*Godness of fit*	*Tajima's D*	*Fu's Fs*
Northern Group	*TOS-CALwest*	0.00(P=0.543)	-1.726(P=0.025)	-4.00(P=0.000)
Southern Group	*PUG-CALeast*	0.06(P=0.112)	0.632(P=0.805)	-1.552(P=0.312)
Clade-A	*TOS-PUG-CAL clade-A*	0.01(P=0.371)	-1.297(P=0.102)	-5.986(P=0.000)
Clade-B	*TOS-PUG-CAL clade-B*	0.04 (P=0.315)	-1.838(P=0.027)	-3.357(P=0.014)

Significance at P<0.05

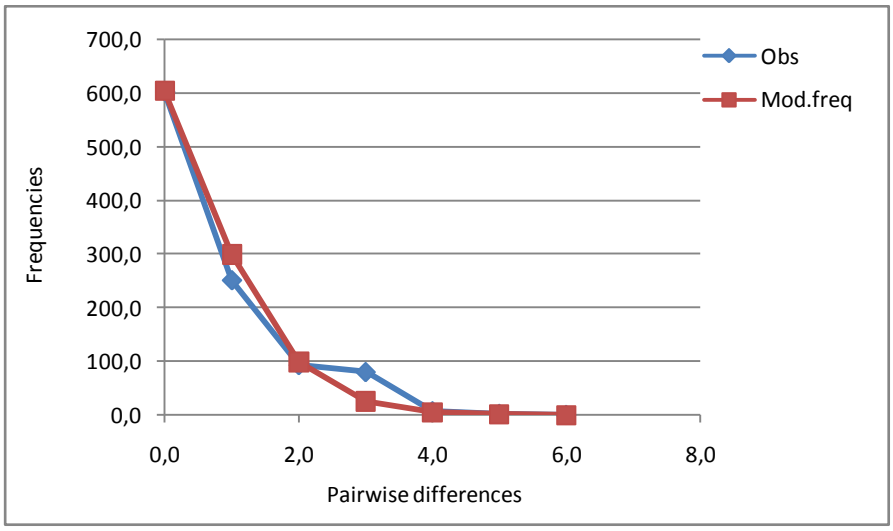

Figure 23: Mismatch distribution of the Northern Group (TOS-CALwest populations) of *H. trunculus*.

The plot representing the Southern Group, does not fit the sudden expansion model, indicated as well by the neutrality tests (Table 11, Figure 24). However the mismatch distributions of the clade-A and clade-B show two genetic pools fitting the sudden expansion model (data not shown). The neutrality tests of both clades are negative and significant (Table 15).

The calculated time of expansion (*t*) of the clade B was 53900 years ago (2.4/million years; 1 generation/year), while the time of expansion (*t*) of the clade A is 26900 years (μ=2.4/million years; 1 generation/year).

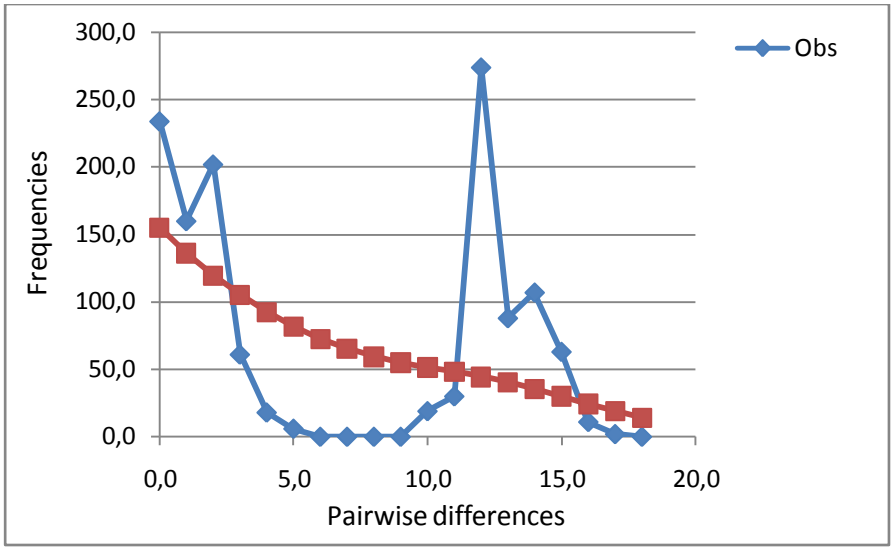

Figure 24: Mismatch distribution of the Southern Group (PUG-CALwest populations) of *H. trunculus*.

5. Discussion

In the present study, were analyzed the genetic structure of two common rocky benthic species, *Halocynthia papillosa* and *Hexaplex trunculus*, in three areas along the Italian coasts. The study areas are located in with the biogeographical breaks in the distribution of species, where the presence of barriers to gene flowhas been hypothesized. The sequencing analysis of mitochondrial gene COI of *Halocynthia papillosa* and *Hexaplex trunculus* populations showed two different genetic structures at the largest scale (ca 1000 km), as well as different demographic history patterns, related to past history events acting differently on the two species. Whereas *H. papillosa* genetic structure showed small but significant differences among regions, in the area of Calabria *Hexaplex trunculus* populations showed a sharp genetic break, probably due to a barrier to gene flow located between Tyrrhenian and Ionian Seas.

At small spatial scale, from about 100 km to 300 km, absence of substructuring has been observed, and hence gene flow, in spite of their different reproductive modes. The meaning, the strengths and the weaknesses, as well as the practical application of these results have been discussed.

5.1.Barriers to gene flow

No evidences of barriers to gene flow were detected in the hypothesized areas of Tuscany and Puglia, neither for *Halocynthiapapillosa*nor for *Hexaplextrunculus*. The analyses of genetic diversity within each location indicate a good level of polymorphism and hence a good level of gene flows within populations. The high number of haplotypes as well as presence of private haplotypes could be an indication of growing or expanding populations (Watterson, 1984). The absence of significant differences across the sides of the hypothetical barriers, according to F_{st} values indicate that for both species gene flow is effective at small distances (100-200 km). The lack of genetic structure at these distances indicates that dispersion occurs between populations despite being located on the limits of some of the biogeographic sectors in the Central Mediterranean (Beckers et al., 1997). These biogeographic sectors are describedon literature according with their hydrogeographical characteristics,which can be variable along the year and hence their influence on

connectivity patterns could change, not conforming a barrier to gene flow for all species (White et al., 2010).

According to the results, the boundaries of the biogeographical regions, as have been defined by Bianchi (2007)do not correspond to effective barriers to gene flow, at least in the case of the two widespread species studied. This could be due to the failure to detect the barrier, given the small scale taken into account, or to the absence of effective barriers in these areas. The lack of genetic structure at small distances could be due to the limited power of COI to detect populations' subdivision respect to other more variable markers. As it has been shown in the bath sponge *Spongiaofficinalis* sampled in 11 locations in the eastern and western Mediterranean and across the Strait of Gibraltar. Whereas genetic divergence between basins could be detected with the mtDNA structure, the existence of subpopulations inside each basin was only detected by means of hyper variable nuclear markers (Dailianis et al., 2011). Although it would be interesting to check the structure given by more variable markers, the main scope of this thesis was to detect long-term barriers to gene flow, shaping the current genetic structure of the species. Furthermore, it has been shown in several instances that genetic structure can be detected along small geographical distances by means of mitochondrial markers. In the Ophiurid *Ophiodermalongicauda*, COI evidenced different genetic pools inside the eastern Mediterranean basin along distances of around 300 km (Boissin et al., 2011), or in the solitary ascidian *Pyura gibbosa* in southern Australia through scales up to 200 km (Ayre et al., 1997). Furthermore, the levels of diversity and polymorphism of the COI gene of the two species studied makes us think that it is suitable to detect substructuring, if present, at the spatial scales considered.

The exception to this pattern is shown by *Hexaplextrunculus*in Calabria. The F_{st}values between the populations at both sides of Calabria and the absence of an isolation by distance pattern, according to the non significant Mantel test, indicate the presence of a genetic break between these nearby populations (about 200 kilometres). Not many examples of such a small scale genetic differentiation have been described in this area: the genetic pattern of *Posidonia oceanica* sampled along the strait of Messina indicate differences between western and eastern Calabria populations suggesting that the western/eastern Mediterranean transition area could be reflected also on the Strait of Messina (Serra et al., 2010).

Other examples regard the whole western/eastern Mediterranean transition: sea bass populations sampled at small distances (about 500 km) along the western/eastern Mediterranean basins revealed genetic structure among them (Bahri-Sfar et al., 2000); the prawn *Penaeuskerathurus,* showed a significant genetic differentiation at both sides of the Siculo-Tunisian Strait among populations located at a maximum distance of 300km (Zitari-Chatti et al., 2009). The genetic break between western andeasternMediterranean, usually defined in the Siculo-Tunisian Strait, has been described for species with diverse life history traits, as bivalves (Quesada et al. 1995; Nikula& Väinölä 2003), several fish species (Borsa et al. 1997; Bahri-Sfar et al. 2000), and seagrasses (Arnaud-Haond et al. 2007). Similar patterns are found across other known barriers to gene flow in the Mediterranean, as in the Ibiza Channel and the Balearic front. This is the case of the crab *Liocarcinus depurator*(Schunter et al., 2011) and the red gorgonian *Paramuriceaclavata*(Mokhtar-Jamaï et al., 2011). The area of the Siculo-Tunisian Strait and the Strait of Messina, as previously stated, is together with the Almeria-Oran front and the Bosphorus Strait among the most important biogeographical barriers of the Mediterranean Sea.

Regarding *H. papillosa* this barrier to gene flow seems to not strongly influence connectivity, maintaining gene flow among distant regions, as far as Toscana and Puglia. This is an interesting pattern, and could be related to the superficial currents crossing the strait of Messina all year long, and that flow principally from west to east (IstitutoIdrograficodella Marina, Genova 1982; Astraldi et al., 1999). However, some works describe a medium and deep opposite water flow co-occurring by upwelling mechanisms, which could pass through the Sicily Strait from east to west and north through the Tyrrhenian Sea (Béranger et al., 2004). This complex annual current regime around the area could be responsible of the admixture of the larvae of *Halocynthiapapillosa* at both sides of Calabria, and the similarity between Toscana and Puglia populations

In the opposite case, *Hexaplextrunculus*connectivity might be restricted by the currents regime, maintaining a geographical separation of the two clades. However, whereas this barrier seems to be strong avoiding the flow from Puglia to Toscana, higher flow exists from Toscana to Puglia as evidenced by the clade-B, present in the Ionian (Calabria east and Puglia west), Southern Adriatic (Puglia east) and Toscana (Toscana north and south) populations, whereas the clade-A, is only present in Puglia (Puglia west and east) and Calabria east populations.

Unidirectional flows have also been recorded across the Almeria-Oran front (AOF) for some fish species, as it seems to promote higher gene flow from west to east (Patarnello et al., 2007). The Ibiza Channel, considered a genetic break for *Serranus cabrilla*, allows a unidirectional flow north/southward that utterly mixes with the Atlanto-Mediterranean main flow. This is confirmed by genetic similarities among Spanish individuals with Mediterranean individuals (Schunter et al., 2011). These unidirectional barriers can follow seasonality changing intensity and direction, influencing the pattern of connectivity of similar species as occurs for *Mytilus galloprovincialis* and *M. californianus*along the coast of California (Carson et al., 2010). Different reproductive seasons could explain the different connectivity patterns of *H. papillosa*and *H. trunculus*.

5.2.Influence of life history traits

Differences in patterns of population genetic structure between the two investigated species could be related to differences in larval dispersal potential. The lack of strong population structure of *Halocynthiapapillosa* can be due to characteristics of the tadpole larva, having a chordate and muscled tail able to swim in the water column during up to one week (Zeng et al., 2006; Kim et al., 2012). For that reason, it is likely to imagine how larvae of this species should be able to search an ideal habitat to settle or to swim far from coastal habitat and encounter the general current systems (Šantić et al., 2010). On the contrary, *Hexaplextrunculus*is considered a short dispersal species, spending a maximum of 24 hours in the water column as juvenile (Lahbib et al., 2010). Moreover, juveniles do not have a good swimming capacity and right after hatching they are ready to settle on the substrata (Lahbib et al., 2010).

However, differences between larval characteristics can't fully explain the genetic pattern of these species. In fact, *H. trunculus* has low levels of genetic differentiation within the two distinct groups separated by the previously described barrier, although the distance included in each group is more than three hundred kilometers. Sea currents can amplify the larval exchange between close and distant populations of low dispersal species, such as the subtidal whelk *Kelletia kelletii*between US and Mexico Pacific coasts (White et al., 2010).

At the same time many authors claim that the mobile larvae of solitary ascidians use their capacity to stay close to the natal substrata, reducing their

dispersal potential (Graham &Sebens, 1996). Many species with high mobility and long larval phases show strong population genetic structure at large scales, such as the spiny lobster *Palinurus elephas*sampled around Europe (Knutsen et al., 2003), and at small distances such as the cod *Gadus morua* sampled at distances of 300 km (Palero et al., 2008). The resulting picture indicates that, both species exhibit a good dispersal capacity through distances of up to 500 km.

There might be henceothers factors influencing the pattern of populations' differentiation observed. An important factor that might influence the different behavior of these two species in the Calabria areacould be the spawning seasonality. *Halocynthiapapillosa* reproduces once per year in late summer (Šantić et al., 2010), whereas *Hexaplextrunculus* reproduces during spring (Abidli et al., 2012). Differences in isothermal regime between spring and late summer and the intensities of currents can influence the dispersal of these two species differently (Astraldi et al., 1999). Many studies demonstrate how in most cases life history traits are not the main drivers of the genetic structure (Weersing and Toonen, 2009): for instance three species of limpets sampled along the Hawaiian archipelago showed three different genetic structures although sharing similar life history traits (Bird et al., 2007). On the other hand, very different life history traits can show very similar genetic structures. Among these cases, that of the kelp bass *Paralabrax clathratus*, Kellet's whelk *Kelletia kelletii* and the California spiny lobster *Panulirus interruptus*, in the Southern California Bight (Toonen et al., 2011).

The present experimental design, where the two species were sampled in the same localities, allows stating that contrasting reproductive mode is not reflected in the structure of the COI gene, and hence in a large temporal scale, distant populations are connected between them.

5.3.Genetic structure and diversity

*Halocynthia papillosa*genetic structure shows little but significant pairwise differencesamong regions, showing a good level of gene flow. Moreover, Toscana and Puglia seem to be more similar compared to Calabria This pattern could be due to the differences in sample size of Calabria respect to the other regions. Another possible explanation could be that local dynamics influence the dispersal of individuals in that region (Bianchi, 2007). The current regime in the coast of

Calabria is not well described on literature but some authors speak about strong currents crossing the strait of Messina and harboring an area characterized by local endemisms and Pleistocene Atlantic remnants (Fredj & Giaccone, 1995).

According to the AMOVA analysis, most of the genetic variability is found within populations (94.72%; P=0.005) but also between regions (4.99%; P=0.078). This slight population structure among regions is not significantly related to geographic distance, indicating probably other factors influencing the gene flow between the three regions.

The exchange of individuals seems bidirectional and asymmetric. In fact, the migration rate from Calabria to Puglia is much higher than in the opposite direction and the same happens from Puglia to Tuscany. The first migration rate could be in agreement with the principal Atlanto-Mediterranean circulation, going from west to east (Hamad et al., 2005). The migration rate between Puglia and Toscana could be related to the deep currents, flowing from the Eastern Mediterranean basins to Tyrrhenian Sea described above. In all, genetic connectivity of *H. papillosa* between areas is high.

Regarding *Hexaplextrunculus*, we found genetic structure between the two groups of populations separated in Calabria as confirmed by AMOVA (17.41%; P=n.s.). Although there are clear evidences of a genetic break, among these groups a certain gene flow seems to exist, given F_{st} values between groups, and the presence of the two lineages in the Southern Group. The migration flow seems to go from the Tyrrhenian Sea to the Ionian Sea. The unidirectional migration flow is observed for some invertebrate species along the AOF (Schunter et al., 2011), along Californian coast (Carson et al., 2010) and across the Isthmus of Panama (McCartney et al., 2000).

The existence of two very distinct genetic pools, one of them found only in Puglia and Calabria east individuals, and the other widespread, cannot be explained by the currents regime, but to past history events, namely vicariance, ancient segregation of populations followed by a posterior mixture of genetic pools (Templeton et al., 1995).

5.4.Influence of past history events

The two species show very different haplotype distributions and demographic history patterns. *H. papillosa*appears as one single genetic pool across the three regions. It fits a pattern of sudden expansion, both for the whole data set as for each of the regions studied. This is confirmed by neutrality tests, negative and significant, indicating population expansion. The haplotype network shows several common haplotypes, with little differences between them and presenting all three regions. A similar pattern was observed by Marko et al. (2010) in some invertebrate species, as *Mytilustrossulusus, Semibalanuscariosus,* and*Balanus glandula*. The time of expansion was calculated in 17700 years ago, around the last glacial maximum of Pleistocene, about 20000 years ago (Huntley & Birks, 1983). This suggests, as explained by Marko et al., (2010), a pattern of persistence during glacial maxima (LGM), about 17700 years ago, and posterior remixing of pools, as seen by several common haplotypes shared through the whole study area.

Hexaplextrunculus demographic history analyses show a clear pattern of two genetic pools separated by a great number of mutations. The haplotype network, mismatch distribution, and neutrality tests confirm that both clades, with their star-like shape, a common haplotype surrounded by little differentiated private haplotypes, represent two expanding lineages (Rogers & Harpending, 1992). In Toscana and Tyrrhenian Calabria only individuals of the clade-B are found, while in Puglia and Ionian side of Calabria, there are individuals belonging to both clades suggesting that these last seem to be more diverse than Toscana (this is also evident on haplotype and nucleotide diversity values).

This pattern is probably due to the LGM, which reduced this species to two glacial refugia and, through vicariance, evolved in the two clades observed. The time of expansion of the clade-A is approximately 53900 years ago, before the LGM, and 26900 years ago for the clade-B, around the LGM. A realistic scenario could be that the clade-A, constituted the older clade and that after the last glacial maximum the two clades were separated. After the LGM is possible that a low level of gene flow was maintained from Tuscany to Puglia, as suggested by the migration rates. Similar patterns are found on the common shrimp *Crangoncrangon* inside the Mediterranean Sea, where phylogroups derive not

only of current physical breaks but also due to populations' segregation during Pleistocene climatic oscillations (Luttikhuizen et al., 2008).

5.5.Multispecies approach

From the results two different patterns of connectivity related both to species-specific traits and to some physical factors were found.

In the light of the results obtained, considerations based on a single species could change the perception of the real extent of barriers and levels of genetic connectivity along the Italian peninsula (Eble et al., 2009). In this area occurrence of contrasting effect of barriers in co-distributed specieshave been shown. Whereas the sand goby *Pomatoschistus minutus* (Stefanni et al., 2003) is well differentiated at both sides of the western/eastern Mediterranean barrier, the prawn *Penaeus kerathurus*shows no structure related to this barrier (Zitari-Chatti et al., 2009).

One of the most recent and relevant example of the multispecies approach is that of Kelly & Palumbi (2010), where 50 species of Pacific nearshore invertebrates were taken into account with the aim of discovering the most influential subtructuring factors along the Pacific coast of North America. This work shows occurrence of similarities and divergences in genetic structure patterns among related and unrelated species at a variety of spatial scales.

It would be interesting to increase our data set with genetic including some other species with different life histories (Bird et al., 2007), following the sampling design as close as possible.

5.6.Management applications

The different genetic patterns of the studied species suggest that a series of complex and linked factors act along the Italian peninsula and that a generalized conservational plan based on one species would not be effective. Relying on pairwise differences values, *Halocynthiapapillosa* shows three connected units, whereas *Hexaplextrunculus* show two highly differentiated but connected units. Following these results two different management plans would be required. Bird et al., (2007) on their study on three species of the genus *Cellana* along the Hawaiian archipelago suggested the implementation of strategies incorporating the differences in population connectivity among species. On conservational

studies it is important taking into account the migration rate between populations in order to approximately infer the populations that could serve as source of migrants for other populations (Eble et al., 2009): while *H. papillosa* shows a bidirectional flow stronger from Calabria to Puglia and from Puglia to Toscana, *H. trunculus* shows an almost unidirectional flow from Toscana to Puglia.

Although the number of species studied does not allow making more complete inferences about the real level of connectivity among these areas, the addition of new data on other common Mediterranean species would help to understand the extent of the barriers to gene flow and connectivity patterns among the Mediterranean basins.

6. Conclusions

The analysis of the population genetic structure and demographic history of *Halocynthiapapillosa* and *Hexaplextrunculus*at different spatial scales by means of the COI mitochondrial gene, allowed us to recognize that neither of the two species shows major genetic break across the putative biogeographical barriers located in Toscana and Puglia. The two species show similar genetic structure at small distances of ca 100-200 km in these two areas, showing a good level of gene flow. However, in the area of Calabria, whereas *H.papillosa* shows no genetic differentiation across eastern and western populations, *H.trunculus* shows a sharp genetic break likely due to the presence of a barrier to gene flow in this area.

Differences in larval dispersal potential between the two species seem not enough to explain the observed different patterns. Their effective larval dispersal seems to be comparable at distances of up to 300 km, as shown by the lack of strong genetic structure between Tuscany and western Calabria populations. Differences in reproductive season might explain why the barrier to gene flow does not affect both species.

Genetic structure at the largest spatial scale shows that populations of *H. papillosa*belong to a single genetic pool with bidirectional migration rates along the whole study area. Conversely, *H. trunculus* populations, are formed by two highly differentiated genetic pools, showing an almost unidirectional migration rates from northern to south-eastern populations.

Demographic history analysis, together with expansion time estimates, indicate that sea level oscillations of the Pleistocene glacial period provoked the vicariance of the two genetic pools of *H. trunculus*, whereas *H. papillosa*probably reduced its populations to a single area. The following expansion processes resulted in the present day structure.

Additional data on other species will allow corroborating the connectivity patterns observed along the Italian coasts. It was possible to recognize different biodiversity units of the two species studied and it should be taken into account in future management and conservational strategies.

7.References

- Abidli S, Lahbib Y & El Menif N T (2012) Relative growth and reproductive cycle in two populations of *Bolinus brandaris* (Gastropoda: Muricidae) from northern Tunisia (Bizerta Lagoon and small Gulf of Tunis). Biologia 67: 751—761
- Astraldi M, Balopoulos S, Candela J, Font J, Gacic M, Gasparini G P, Manca B, Theocharis A, Tintore J (1999) The role of straits and channels in understanding the characteristics of Mediterranean circulation. Progr in Ocean 44: 65–108
- Avise J C (2004) Molecular markers, natural history, and evolution. Sec Ed 541:30-47, 87-98, 231-316
- Ayre D J, Minchinton T E, Perrin C (2009) Does life history predict past and current connectivity for rocky intertidal invertebrates across a marine biogeographic barrier? Mol Ecol 18: 1887–1903
- Bahri-Sfar L, Lemaire C, Hassine O K B & Bonhomme F (2000) Fragmentation of Sea Bass populations in the Western and Eastern Mediterranean as revealed by microsatellite polymorphism. Biol Sci 267: 929-935
- Bandelt H J, Forster P & Röhl A (1999) Median-Joining networks for inferring intraspecific phylogenies Mol Biol Evol 16: 37-48
- Barber P H, Palumbi S R, Erdmann M V, MoosaM K (2000) A marine Wallace's line? Nature 406: 238-256
- Bazin E, Glémin S, Galtier N (2006)Population size does not influence mitochondrial genetic diversity in animals. Science 312: 570
- Beckers J M, Brasseurt P, & Nihoul J C J (1997) Circulation of the western Mediterranean: from global to regional Scales. Deep-Sea Res 44: 531-549
- Beerli P, Felsenstein J (1999) Maximum likelihood estimation of migration rates and effective population numbers in two populations using a coalescent Approach. Genetics152: 763–773
- Béranger K, Mortier L, Gasparini G P, Gervasio L, Astraldi M, Crépon M (2004) The dynamics of the Sicily Strait: a comprehensive study from observations and models. Deep-Sea Res 51: 411–440
- Bertness M D, Gaines S D, & Hay M (2001)*Marine community ecology*.Sinauer Associates Inc 13: 339-366, 18: 469-508, 19: 509-530
- Bianchi C N (2007) Biodiversity issues for the forthcoming tropicalMediterranean Sea. Hydrobiologia 580: 7–21

- Bird C E, Holland B S, Bowen B W & Toonen R J (2007) Contrasting phylogeography in three endemic hawaiian limpets (*Cellana spp*.) with similar life histories. Mol Ecol 16: 3173–3186
- Boissin E, Stöhr S & Chenuil A (2011) Did vicariance and adaptation drive cryptic speciation and evolution of brooding in *Ophioderma longicauda* (Echinodermata: Ophiuroidea), a common Atlanto-Mediterranean ophiuroid? Mol Ecol 2: 4737–4755
- Borrero-Pérez G H, González-Wangüemert M, Marcos C & Pérez-Ruzafa A (2011) Phylogeography of the Atlanto-Mediterranean sea cucumber *Holothuriamammata* (Holothuria): the combined effects of historical processes and current oceanographical pattern. Mol Ecol 20: 1964–1975
- Borsa P, Blanquer A, Berrebi P (1997) Genetic structure of the flounders *Platichthys flesus* and *P stellatus* at different geographic scales. Mar Biol 129: 233-246
- Botsford L W, White J W, Coffroth M-A, Paris C B, Planes S, Shearer T L, Thorrold S R, Jones G P (2009) Connectivity and resilience of coral reef metapopulations in marine protected areas: matching empirical efforts to predictive needs. Cor Reefs 10: 338-466
- Brasseur P, Beckers J M, Brankart J M & Schoenauen R (1996) Seasonal temperature and salinity fields in the Mediterranean Sea: climatological analyses of an historical data set. Deep Sea Res 42: 159–192
- Calderon I, Giribet G, Turon X (2008) Two markers and one history: phylogeography of the edible common sea urchin *Paracentrotus lividus* in the Lusitanian region. Mar Biol 154: 137-151
- Carpenter K E, Barber P H, Crandall et al., (2011) Comparative phylogeography of the coral triangle and imlications for marine management. J Mar Biol 2011: 1-14
- Carson HS, Lòpez-Duarte PC, Rasmussen L, Wang D, Levin LA (2010) Reproductive timing alters population connectivity in marine metapopulations. C Biology, 20: 1926-1931
- Carstens B C, Stoute H N & Reid N M (2009) An information-theoretical Applroach to phylogeography. Mol Ecol 18: 4270–4282
- Caswell H (1976) Community structure–neutral model analysis. Ecol Monogr 46: 327–354

- Cerrano C, Ponti M, Silvestri S (2004) Guida alla biologia marina del Mediterraneo. Ananke 51: 81-92, 513: 203-254

- Chao A & Shen T-J (2010) Program SPADE (Species Prediction And Diversity Estimation) Program and User's Guide published at http://chaostat.nthu.edu.tw/
- Clarke K R & Ainsworth M (1993) A method of linking multivariate community structure to environmental variables. Mar Ecol Prog Ser 92: 205-219
- Claudet J, et al. (2008) Marine reserves: size and age do matter. Ecol Let 11: 481-489
- Coll M, Piroddi C, Steenbeek J, Kaschner K, Lasram B R F, et al. (2010) The biodiversity of the Mediterranean Sea: estimates, patterns, and threats. PLoS ONE 5: e11842
- Coma R, Ribes M, Gili J M, Zabala M (2002) Seasonality of in situ respiration rate in three temperate benthic suspension feeders.Limnol Oceanogr 47: 324-331
- Cunningham C W & Collins T M (1998) Beyond area relationships: extinction and recolonization in molecular marine biogeography. R De Salle, B Schierwater (eds), Mol Appl Ecol Evol 297-321
- Dailianis T, Tsigenopoulos C S, Dounas C & Voultsiadou E (2011) Genetic diversity of the imperilled bath sponge *Spongia officinalis* Linnaeus, 1759 across the Mediterranean Sea: patterns of population differentiation and implications for taxonomy and conservation. Mol Ecol 20: 3757–3772
- Demarchi M, ChiApplero M B, Sahade M T R (2010) Population genetic structure of the Antarctic ascidian *Aplidium falklandicum*from Scotia Arc and South Shetland Islands.Polar Biol 33: 1567–1576
- Di Camillo C G, Coppari M, Bartolucci I, Bo M, Betti F, Bertolino M, Calcinai B, Cerrano C, De Grandis G, Bavestrello G (2012) Temporal variations in growth and reproduction of *Tedania anhelans* and *Chondrosia reniformis* in the North Adriatic Sea. Hydrobiologia 687: 299–313
- Durante K M & Sebens K P (1994) Reproductive ecology of the ascidians *Molgula citrina* Alder & Hancock, 1848 &*Aplidium glabrum* (Verrill, 1871) from the Gulf of Maine. USA Ophelia 39: 1-21
- Eble J A, Toonen R J, Bowen B W (2009) Endemism and dispersal: comparative phylogeography of three surgeonfishes across the Hawaiian Archipelago. Mar Biol 156: 689–698

- Excoffier L & Lischer H E L (2010) Arlequin suite ver. 35: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res 10: 564-567
- Folmer O, Black M, Hoeh W, Lutz R & Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome coxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3: 294-299
- Fu Y X (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics147: 915–925
- Graham K R &SebensK P (1996) The distribution of marine invertebrate larvae near vertical surfaces in the rocky subtidal zone. Ecol 77: 933-949
- Hamad N, Millot C, Taupier-Letage I (2005)A new hypothesis about the surface circulation in the easternbasin of the Mediterranean sea. Progr Ocean 66: 287–298
- Hannan C A (1984) Planktonic larvae may act like passive particles in turbulent near-bottom flows. Lim & Ocean 29: 1108-1116
- Harpending H C (1994) Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. Human Biol 66: 591-600
- Hart M W & Marko P B (2010) It's about time: divergence, demography, and the evolution of developmental modes in marine invertebrates. Int Com Biol 50(4): 643–661
- HellbergM E & Vacquier V D (1999) Rapid evolution of fertilization selectivity and Lysin cDNA sequences inTeguline gastropods. Mol Biol Evol 16: 839–848
- Hirano T, Nishida H (2000) Developmental fates of larval tissues after metamorphosis in the ascidian, *Halocynthia roretzi.* Dev Genes Evol 210: 55–63
- Hofrichter R (2001) El mar Mediterráneo (Fauna Flora Ecología). I Parte general. Ed Omega 5: 258-287, 6: 356-383, 8: 464-499
- Horne J B, Herwerden L V, Choat J H, Robertson D R (2008) High population connectivity across the Indo-Pacific: congruent lack of phylogeographic structure in three reef fish congeners. Mol Phyl Evol 49: 629–638
- Huntley B, Birks H J B (1983) An atlas of past and present pollen maps for Europe: 0-13000 years ago. Cambridge University Press Cambridge 17: 239-257
- Jackson D, Leys S P, Hinman V F, Woods R, Lavin M F & Degnan B M (2002) Ecological regulation of development: induction of marine invertebrate metamorphosis. Int J Dev Biol 46: 679-686

- Jackson J B C (1986) Modes of dispersal of clonal benthic invertebrates: consequences for species distributions and genetics structure of local populations. Bul Mar Sci 39: 588-606
- Kalkan E, Kurtuluş A, Maraci Ö & Bilgin R (2011) Is the Bosphorus Strait a barrier to gene flow for the Mediterranean mussel, *Mytilus galloprovincialis* (Lamarck, 1819)? Mar Biol Research 7: 690-700
- Kelly R P, Oliver T A, Sivasundar A, & Palumbi S R (2010) A Method for detecting population genetic structure in diverse, high gene-flow species. J Hered 101: 423–436
- Kelly R P, Palumbi S R (2010) Genetic structure among 50 Species of the Northeastern Pacific rocky intertidal community. PLoS ONE 5: e8594
- Kim G J & Nishida H (1998) Monoclonal antibodies against differentiating mesenchyme Cells in larvae of the ascidian *Halocynthia roretzi.*Zool Sci 15: 553–559
- Knutsen H, Jorde P E, Andre C, Stenseth N C (2003) Fine-scaled geographic population structuring in a highly mobile marine species: the Atlantic cod. Mol Ecol 12: 385-39
- Kruskal J B (1956) On the shortest spanning subtree of a graph and the traveling Salesman problem.Proc American Mat Soc 7: 48–50
- Lahbib Y, Abidli S, & El MenifN T (2011) Reproductive activity in the commercially exploited MediterraneanMuricid *Hexaplex trunculus*collected from Boughrara Lagoon(Southern Tunisia). Russian J Mar Biol 37: 501–508
- Larson A, Wake D B&YanevK P (1984) Measuring gene flow among populations having high levels of genetic fragmentation. Genetics106: 293-308
- Launey S, Ledu C, Boudry P, Bonhomme F & Naciri-Graven Y (2002)Geographic structure in the European flat oyster (*Ostrea edulis*L) as revealed by microsatellite polymorphism J Hered93:331-351
- Lazoski C, Solé-Cava A M, Esnault N B, Klautau M, Russo C A M (2001) Cryptic speciation in a high gene flow scenario in the oviparous marine sponge *Chondrosia reniformis*. Mar Bio 139: 421-429
- Leng L & Zhang De-X (2011) Measuring population differentiation using GST or D? A simulation study with microsatellite DNA markers under a finite island model and nonequilibrium conditions. Mol Ecol 20: 2494–2509

- Leys S P & Ereskovsky A V (2006) Embryogenesis and larval differentiation in sponges.Can J Zool 84: 262-287
- Librado P & Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data.Bioinformatics25: 1451-1452
- López-Legentil S & Turon X (2006) Population genetics, phylogeography and speciation of*Cystodytes*(Ascidiacea) in the western Mediterranean Sea. Biol J Linn Soc 88: 203-214
- Luttikhuizen P C, Campos J, Van Bleijswijk J, Peijnenburg K TCA, Van der Veer H W (2008) Phylogeography of the common shrimp, *Crangonn crangon* (L) across its distribution range. Mol Phyl Evol 46: 1015-1030
- Mantel N (1967) "The detection of disease clustering and a generalized regression Applroach". Cancer Res27: 209–220
- Mariani S, Ketmaier V, de Matthaeis E (2002) Genetic structuring and gene flow in *Cerastoderma glaucum* (Bivalvia: Cardiidae): evidence from allozyme variation at different geographic scales. *Mar Biol*, 140: 687–697
- Marko P B, Hoffman J M, Emme S A, McGovern T M, Keever C C & Cox L N (2010) The 'Expansion–Contraction' model of Pleistocene biogeography: rocky shores suffer a sea change? Mol Ecol 19: 146–169
- Miller M P, Bellinger M R, Forsman E D, & Haig S M (2006) Effects of historical climate change, habitat connectivity, andvicariance on genetic structure and diversity across the rangeof the red tree vole (*Phenacomys longicaudus*) in the PacificNorthwestern United States. Mol Ecol 15: 145–159
- Millot C (1992) Are there major differences between the largest Mediterranean Seas? A preliminary investigation Bulletin de l' Institut Océanographique 11: 3–25
- Mokhtar-Jamaï K, Pascual M, Ledoux J-B et al. (2011) From global to local genetic structuring in a red gorgonian *Paramuricea clavata*: the interplay between oceanographic conditions and limited larval dispersal. Mol Ecol 20: 3291-3305
- Muths D, Jollivet D, Gentil F, Davoult D (2009) Large-scale genetic patchiness among NE Atlantic populations of the brittle star *Ophiothrix fragilis.* Aquat Biol 5: 117-132
- Nikula R, Väinölä R (2003) Phylogeography of *Cerastoderma glaucum* (Bivalvia: Cardiidae) across Europe: a major break in the Eastern Mediterranean. Mar Biol 143: 339–350

– Palero F, Abello P, Macpherson E, Gristina M, Pascual M (2008) Phylogeography of the European spiny lobster (*Palinurus elephas*): influence of current oceanographical features and historical processes. Mol Phyl & Evol 48: 708-717
– Patarnello T, Volckaert F A M J & Castilho R (2007) Pillars of Hercules: is the Atlantic–Mediterranean transitiona phylogeographical break? Mol Ecol 16: 4426–4444
– Peijnenburg K T C A, Fauvelot C, Breewer J A J & Menken S B J (2006) Spatial and temporal genetic structure of the planktonic*Sagitta setosa*(Chaetognatha) in European seas as revealedby mitochondrial and nuclear DNA markers. Mol Ecol 15: 3319–3338
– Pérez-Portela R, Turon X, Bishop J D D (2012) Bottlenecks and loss of genetic diversity: spatio-temporal patterns of genetic structure in an ascidian recently introduced in Europe. Mar Ecol Prog Ser 451: 93–105
– Pérez-Portela R, Villamor A, Almada V (2010) Phylogeography of the sea star *Marthasterias glacialis* (Asteroidea, Echinodermata): deep genetic divergence between mitochondrial lineages in the north-western Mediterranean. Mar Biol 157: 2015–2028
– Perry R I, Cury P, Brander K, Jennings S, Möllmann C, Planque B (2010) Sensitivity of marine Systtems to climate and fishing: concepts, issues and management responses. J Mar Syst 79: 427–435
– Pluzhnikov A & Donnelly P (1996)Optimal sequencing strategies for surveying molecular genetic diversity. Genetics144: 1247-1262
– Ponti M, Fava F, Abbiati M (2011) Spatial–temporal variability of epibenthic assemblages on subtidal biogenic reefs in the northern Adriatic Sea. Mar Biol 158: 1447–1459
– Ribes M, Coma R, Gili J M (1998) Seasonal variation of in situ feeding rates by thetemperate ascidian *Halocynthia papillosa*.Mar Ecol Prog Ser 175: 201-213
– Riesgoa A & Maldonado M (2008) Differences in reproductive timing among sponges sharing habitat and thermal regime. InvertebrBiol 127: 357–367
– Rizzo C, Cammarata M, Di Carlo M, Pancucci A & Parrinello N (2009) RAPD Profiles Distinguish *Paracentrotus lividus* Populations living in a stressing environment (Amvrakikos Gulf, Greece). Russ J Gen 45: 499-503
– Rocklin D, Tomasini J A, Culioli J M, Pelletier D, Mouillot D (2011) Spearfishing regulation benefits artisanal fisheries: the ReGS indicator and its

Appllication to a multiple-use mediterranean Marine Protected Area. PLoS ONE 6: e23820

- Rogers A R & Harpending H (1992) Population growth makes waves in the distributionof pairwise genetic differences. Mol Bid Evol 9: 552-569
- Roman J & Palumbi S R (2004) A global invader at home: population structure of the green crab, *Carcinus maenas*, in Europe. Mol Ecol 13: 2891-2898
- Rosindell J, Hubbell S P, & Etienne R S (2011) The unified neutral theory of biodiversity and biogeography at age ten trends. Ecol & Evol 26: 340-348
- Russo G F, Fasulo G, Toscano A & Toscano F (1990) On the presence of triton species (*Charonia spp*) (Mollusca, Gastropoda) in the Mediterranean Sea: ecological considerations. Bollettino malacologico 26: 91–104
- Sakairi K & Shirai H (1991) Possible MIS production by follicle cells in spontaneous oocyte maturation of the ascidian, *Halocynthia roretzi*.Dev Gro & Diff 33: 155-162
- Šantić M, Rada B, PaladinA & Pleslić G (2010) The influence of some abiotic parameters on growth inclination in Ascidian *Halocynthia Papillosa*(Linnaeus, 1767) From The Northern Adriatic Sea (Croatia). Arch Biol Sci 62: 1007-1011
- Schreiber K, Hauffe T, Abbrecht C, Wilke T (2012) The role of barriers and gradients in differentiation processes of pyrogulinid microgastropods of Lake Ohrid. Hydrobiologia 682: 61-73
- Schunter C, Carreras-Carbonell J, Macpherson E, Tintoré J, Vidal-Vijande E, Pascual A, Guidetti P & Pascual M (2011) Matching genetics with oceanography: directional gene flow in a Mediterranean fish species. Mol Ecol 20: 5167–5181
- Smith K F, Stefaniak L, Saito Y, Gemmill C E C, Cary S C, Fidler A E (2012) Increased inter-colony fusion rates are associated with reduced COI haplotype diversity in an invasive colonial ascidian *Didemnum vexillum*. PLoS ONE 7: e30473
- Stamatis C, Triantafyllidis A, Moutou K A & Mamuris Z (2004) Mitochondrial DNA variation in Northeast Atlantic and Mediterranean populations of Norway lobster, *Nephropsnorvegicus*. Mol Ecol 13: 1377–1390
- Stefanni S & Thorley J L (2003) Mitochondrial DNA phylogeography reveals the existence of an Evolutionarily Significant Unit of the sand goby *Pomatoschistus minutus* in the Adriatic (Eastern Mediterranean). Mol Phyl & Evol 28: 601-609

- Svane I & Young C M (1989) The ecology and behavior of ascidian larvae. Oceanogr Mar Biol annual review 27: 45-90
- Tajima F (1989) Statistical testing for the neutral mutation hypothesis by DNA polymorphism. Genetics123: 585–595
- Tarjelò I & Turon X (2004) Resource allocation in ascidians: reproductive investment vs other life-history traits. InvertebrBiol 123: 168-180
- Tarjuelo I, Posada D, Crandall K A, Pasqual M, Turon X (2001) Cryptic species of *Clavelina* (Ascidiacea) in two different habitats: harbors and rocky littoral in northwestern Mediterranean. Mar Biol 139: 455-462
- TempletonA R,Routman E & Phillips C A (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. Genetics 140:767-782
- Tintore J, La Violette P E, Blade I, Cruzado A (1988) A study of an intense density front in the Eastern Alboran Sea: the Almeria-Oran Front. J phys Ocean 18: 1384-1397
- Toonen R J, Andrews K R, Baums I B, Bird C E, Concepcion G T, Daly-Engel T S, Eble J A, Faucci A, Gaither M R, Iacchei M, Puritz J B, Schultz J K, Skillings D J, Timmers M A, & Bowen B W (2010) Defining boundaries for EcoSysttem-Based Management: a multispecies case study of marine connectivity across the Hawaiian archipelago. J Mar Biol 2011: 1-13
- Triantafyllidis A, Apostolidis A P, Katsares V, Kelly E, Mercer J, Hughes M, Jorstad K, Tsolou A, Hynes R, Triantafyllidis C (2005) Mitochondrial DNA variation in the European lobster (*Homarus gammarus*) throughout the range. Mar Biol 146: 223-235
- Usher K M & Ereskovsky A V(2005) Larval development, ultrastructure and metamorphosis in*Chondrilla australiensis*Carter, 1873 (Demospongiae, Chondrosida, Chondrillidae). Invertebr Reprod Dev47**:** 51-62
- Vermeij G J (2012) The tropical history and future of the Mediterranean biota and the West African enigma. J Biogeogr 39: 31–41
- Waples R S (1998) Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. J Hered 89: 438–450
- Watterson G A (1984) Allele frequencies after a bottleneck theoretical population. Biol26: 387–407

– Weersing K, Toonen RJ (2009) Population genetics, larval dispersal, and connectivity in marine systems. Mar Ecol Prog Ser 393: 1-12
– White C, Selkoe K A, Watson J, Siegel D A, ZacherlD C& Toonen R J (2010) Ocean currents help explain population genetic structure. Proc R Soc B 277: 1685–1694
– Xavier J R, Van Soest R W M (2012) Diversity patterns and zoogeography of the Northeast Atlantic and Mediterranean shallow-water sponge fauna. Hydrobiologia 687: 107-125
– Zane L, Ostellari L,Maccatrozzo L,Bargelloni L, Cuzin-Roudy J, Buchholz F &Patarnello T (2000) Genetic differentiation in a pelagic crustacean (*Meganyctiphanes norvegica*: Euphausiacea) from the North East Atlantic and the Mediterranean Sea. Mar Biol136: 191-199
– Zarai Z, Frikha F, Balti R, Miled N, Gargouri Y & Mejdoub (2011) H Nutrient composition of the marine snail (*Hexaplex trunculus*) from the Tunisian Mediterranean coasts. J Sci Food Agric91: 1265–1270
– Zardoya R, Castilho R, Grande C, Favre-Krey L, Caetano S, Marcato S, Krey G, & Patarnello T (2004) Differential population structuring of two closely relatedfish species, the mackerel (*Scomber scombrus*) and the chubmackerel (*Scomber japonicas*), in the Mediterranean Sea. Mol Ecol 13: 1785–1798
– Zeng L, Jacobs M W & Swalla B J (2006) Coloniality has evolved once in stolidobranch ascidians. Int Com Biol 46: 255–268
– Zitari-Chatti R, Chatti N, Fulgione D, Caiazza I, Aprea G, Said A E K, Capriglione T, (2009) Mitochondrial DNA variation in the caramote prawn *Penaeus* (Melicertus) *kerathurus* across a transition zone in the Mediterranean Sea. Genetica 136:439–447
– Zulliger D E, Tanner S, Ruch M, Ribi G (2009) Genetic structure of the high dispersal Atlanto-Mediterranean sea star *Astropecten aranciacus* revealed by mitochondrial DNA sequences and microsatellite loci. Mar Biol 156: 597-610

8. Acknowledgements

Un ringraziamento molto speciale e non formale ad
Adriana, Federica e Abbiati
che con il loro aiuto, sostegno e soprattutto pazienza
hanno reso la mia tesi degna di essere letta,
altrimenti non so cosa sarebbe venuto fuori. GRAZIE!

Alla mia famiglia e soprattutto a mio babbo,
sostegno reale e realista in tutta la mia vita e al mio percorso universitario.

A mia mamma,
che anche da lassù non smette di combattere per la mia felicità e la mia fede.

A tutti i miei amici,
che mi hanno sostenuto e sostengono sempre.

Al mare, che mi appassiona da sempre
e mi ricorda la nostalgia dell'infinito di ogni uomo:
"Se vuoi costruire una barca,
non radunare uomini per tagliare legna,
dividere i compiti e impartire ordini,
ma insegna loro la nostalgia per il mare vasto e infinito".
(Antoine de Saint-Exupéry)