ALMA MATER STUDIORUM

UNIVERSITÀ DI BOLOGNA

Facoltà di Scienze Matematiche, Fisiche e Naturali

Laurea Magistrale in Biologia Marina

Genetic structuring of *Eunicella singularis* along a bathymetric gradient

Candidato:

Terrazzoni Licia Giovanna

Relatore:

Prof. Marco Abbiati

Correlatrice:

Dott.sa Federica Costantini

III Sessione

Anno Accademico 2012-2013

Summary

ABSTRACT

Phenotypic plasticity refers to the ability of an organism to express different morphologies depending on the abiotic and biotic environment. Depth integrating many variables (e.g. temperature, light and hydrodynamics), may affect population structure and dynamics of the populations, as well as connectivity patterns and genetic diversity. *Eunicella singularis* is a Mediterranean arborescent gorgonian who plays an important rule as engineer species providing biomass and complexity to coralligenous habitats. It has a wide bathymetric distribution ranging from shallow rocky bottoms to deep sublittoral reefs. The species shows two depth-related morphotypes which taxonomic status is not yet clarified. The aim of the study is to analyse genetic variability and/or structuring along a vertical gradient to test the presence of the two morphotypes. Furthermore, a preliminary analyses of the phylogenetic relationship among species of the genus *Eunicella* has been done. Six populations of *Eunicella singularis* were sampled from 10 to 60 m depth in Cap de Creus and individuals belonging to *Eunicella cavolinii, E. verrucosa, E. racemosa and E. stricta aphyta* were collected. The genetic analyses were carried out using five microsatellite loci and ITS-1 sequence polymorphism. The results showed a reduction of genetic variability along the vertical gradient. A threshold in connectivity was observed across 30 - 40 m depth, confirming the presence of two different *Eunicella singularis* morphotypes. The two morphological forms could be due to phenotypic plasticity, which allowed populations to suit different environmental conditions, or to a break in gene flow that determined the isolation of the two populations and an accumulation of genetic differences. The molecular markers used were not able to clarify the phylogenetic relationship among *Eunicella* species and the systematic position of the two morphotypes, conversely they risen the question on the existence of single species of Mediterranean *Eunicella*.

1 INTRODUCTION

1.1 Phenotypic plasticity, local adaptation and speciation

Phenotypic plasticity refers to how the environment affects the phenotype of an organism within its lifetime (Todd et al. 2008). Species are phenotypically plastic, when during their grown, they respond to the environment changes through changes in morphology (Agrawal 2001). Plasticity is often visualised graphically as a reaction norm, by plotting the different phenotypes expressed as a function of the environment (Figure 1, Schlichting and Pigliucci 1998).

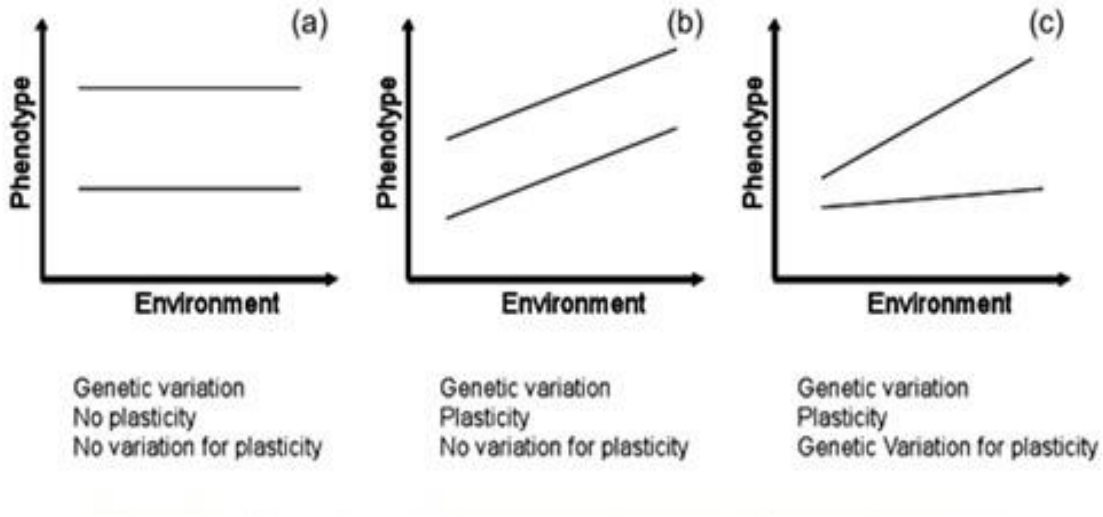

Figure 1. Illustration of phenotypic plasticity using reaction norm of two genotypes. Each line represent one genotype and its phenotypic expression along an environmental gradient. (a) The two genotypes have different phenotypes, but the phenotype is insensitive to the environment, thus no phenotypic plasticity is present. (b) The two genotypes produce different phenotypes in different environment, thus plasticity is present. However the magnitude of plasticity (the slop of the reaction norm) is similar, so there is no genetic variation in plasticity in the population. (c) The two genotypes show different degree of plasticity, thus there is genetic variation for plasticity in the population (i.e. a genotype x environment interaction).

An organism with a particular genotype grows with a determined set of characteristics. In a different environment these characters may remain the same or change and the character's plasticity is measured by the level of the change (Bradshaw 1965). Plasticity is considered adaptive if the phenotypic changes induced by environmental conditions have a clear functional relationship with the environmental signal (see Todd et al. 2008 for a review). This relationship must result in an improvement in fitness, reproduction, growth, or survival (Stearns 1989; Newman 1992). Some authors claim that adaptive phenotypic plasticity must have a historical basis, so populations need repeated exposure to particular environment conditions to evolve a plastic response (Schmalhausen 1986). Many parameters can influence organisms' morphology: light intensity, salinity, depth, sedimentation, geographical distribution, presence of predators, prey availability and so on, all of them determining factors for species' (Yoshioka and Yoshioka 1989; Virgilio et al. 2006). Marine modular organisms, like corals, exhibit the most extensive phenotypic plasticity (see Todd et al. 2008 for a review). Several studies on different modular species, usually based on transplant experiments, showed a wide phenotypic plasticity in a wide range of environments (Schlichting 1986). Differences in water turbulence (Thèodor 1963; Skoufas et al. 2000), degree of wave exposure during growth (Schmidt-Roach et al. 2013), or even attachment orientation with respect to gravity force can greatly modify growth and hence morphology in this kind of organisms. Light intensity can induce more complex branching patterns in some corals as *Porites sillimaniani* (Muko et al. 2000) or *Synaraea convexa* (Jaubert et al.1977) All these abiotic parameters exert their influence on the morphology of modular animals independently, but their effect is more evident when they act concurrently as along environmental gradients (e.g. latitudinal and/or depth gradient). The depth gradient integrates many environmental variables (e.g. temperature, light, hydrodynamics, among others), which may affect population structure and dynamics (Garrabou et al. 2002): for instance shallow colonies of *Eunicella flexuosa*, are exposed to high water motion, so they are taller, developed in a single plane with thicker branches and bigger calices, whereas deeper colonies, subjected to lower water motion, exhibit a multiplane growth with fewer terminal branches and smaller and sparser calices (Prada et al. 2008). The shallow water Caribbean gorgonian coral *Pseudopterogorgia bipinnata*, can be found from

wave-swept environments as shallow as 1 m to about 45 m, where water movement is very low, exhibiting large morphological variability, from the color of the colonies to the branching pattern, with over tenfold difference in branch length towards its environmental extremes (Sanchez et al. 2007). Plasticity is an alternate mechanism for achieving adaptive phenotypes in heterogeneous environments, and in this way it should promote local adaptation (Schmidt-Roach et al. 2013). Local adaptation is defined as the fine-tuning of populations to their local environment via natural selection (Kelly et al 2011). As a result of local adaptation resident genotypes will have a higher fitness in their native habitat than foreign genotypes from distant populations (Kawecki and Ebert 2004). Adaptive differentiation in marine invertebrate populations can be driven by many abiotic and biotic factors (salinity, wave strength, anthropogenic changes, predation intensity, dispersal capacity and gene flow (Kelly et al 2011)). These phenotypic differences may reflect accumulated genetic variation due to disruption of gene flow between populations, which might end up in speciation if the populations are large enough and separated for enough time (Schluter 2001; Gavrilets 2003). However, different selection pressures are not the only factors that can explain phenotypic differences between populations: neutral genetic drift and novel mutations over time can also lead to differentiate populations (Merilä and Crnokrak 2001; Hendry and Taylor 2004). To be able to distinguish between these two processes the differences between populations on a common scale has to be estimated. Population genetics offers a useful way to identify signatures of genetic evolution and local adaptation to environmental change in organisms for which traditional approaches (e.g. transplants; Howells et al. 2013) are impractical. To date, knowledge about the role of gene flow on the evolution of phenotypic plasticity is lacking. Theoretical models (Scheiner 1998; Sultan and Spencer 2002) have identified gene flow between populations as a factor that increases the likelihood of plasticity. Moreover, models also suggest that plasticity by itself can promote dispersal, as plastic individuals are more likely to survive in novel environments with different selection pressures (Price et al. 2003). Studies aimed to understand whether morphological variability of coral colonies is due to phenotypic plasticity or genetic variability showed different patterns: whereas two morphotypes of *Favia fragum* seem to be related to the partial evolution of reproductive barriers, two distinct morphotypes of

the Caribbean octocoral *Briareum asbestinum* presented larger genetic differences, suggesting absent or greatly restricted gene flow between morphs. Ecological barriers to gene flow can ultimately lead to complete reproductive isolation and the formation of new species (Bongaerts et al. 2011). Coral taxonomy is still based on skeletal morphology but, as seen before, these characters presented a great variability within species and are often correlated with environmental conditions (Veron 1986, 2000). This variability has led to considerable confusion regarding species boundaries and classification. A better understanding of phenotypic plasticity has often challenged classical taxonomy in several coral groups (e.g. Miller 1994; Shaish et al. 2007; Todd et al. 2008). For example, *Pocillopora darmicornis* consists of 5 genetically distinct mitochondrial lineages corresponding to five previously reported *P. damicornis sensu lato* morphotypes (Schmidt-Roach et al. 2013). In the opposite, *Pseudopterogorgia bipinnata*, widespread shallow water Caribbean gorgonian, was considered a taxonomic complex due to variable color, morphology and branch size depending on depth and exposition. In this case molecular evidence corroborated that the morphological differences corresponded to phenotypic plasticity (Sanchez et al. 2007).

1.2 Depth effects on population's structure and dynamics

As seen before, the depth, integrating many variables (e.g. temperature, light and hydrodynamics), may affect population structure and dynamics (Garrabou et al. 2002), life-history, trophic ecology, biochemical pathways and physiology. Moreover, depth may also affect connectivity patterns and genetic diversity. Different studies showed that potential for population differentiation may vary with depth (Etter and Rex 1990; France and Kocher 1996). Bongaerts et al. (2011) using a reciprocal transplant experiments, demonstrated that the previously described genetic partitioning of *Seriatopora hystrix* across depth related habitats reflects a process of adaptive divergence along a depth gradient. Mitochondrial and nuclear loci of the coral indicated the occurrence of little to no gene flow between habitat-associated populations. In contrast to the sharp differentiation across adjacent habitats, genetic similarity was observed in similar habitat types at different geographic locations. Costantini et al (2011) reported a consistent reduction in red coral genetic variability

along a depth gradient. These results suggest that depth has an important role in determining the patterns of genetic structure in *Corallium rubrum*. Moreover, a threshold in connectivity was observed among the samples collected across 40–50 m depth, supporting the hypothesis that discrete shallow and deep-water red coral populations occur.

1.3 Molecular tools

In corals, due to the very low mutation rate of mitochondrial DNA, microsatellite loci and nuclear sequences polymorphism (e.g. Internal transcribed spacer – ITS) are the commonly used molecular markers. Microsatellites are short sequences of non-coding DNA highly repeated in tandem and known by two acronyms: SSRs (Simple Sequence Repeats) and STRs (Short Tandem Repeats). They are ubiquitous in prokaryotes and eukaryotes, and in the genome of vertebrates most of them (30–67%) are dinucleotides. SSRs contribute to the DNA structure, chromatin organization, and regulation of recombination, transcription, translation, and gene expression (Chistiakov et al. 2005). Microsatellites are selectively neutral markers, with a mutation rate estimated at 10^{-2}–10^{-6} per locus per generation (Ellegren 2000), which is several orders of magnitude greater than that of regular non repetitive DNA (Li 1997). Therefore, they show high levels of allele polymorphism, and represent a useful tool for population genetic studies in a short time scale. In particular they allow to define the genetic structuring of populations and to estimate larval dispersal capacity of the species. Moreover, they are useful to investigate the genetic relationship among individuals and to evaluate the inbreeding levels within populations (Charlesworth et al. 1997; Reusch et al. 2001).

The Internal Transcribed Spacers (ITS) are non coding regions that separate the 18S, 5.8S and 28S genes which form the ribosomal repeat unit (rDNA) (Hillis & Dixon 1991). The rDNA is a multigene family with nuclear copies in eukaryotes arranged in tandem arrays. These ITS regions have a rapid rate of mutation because they have few selective constraints, making them ideal for comparative analysis at the intra-population and intra-specific level (Caporale et al. 1997). These markers have been used to study population genetics of different tropical coral species (Rodriguez-

Lanetty 2002; Takabayashi et al. 2003; Le Goff-Vitry et al. 2004). They have been used for molecular phylogenetic and taxonomic study in gorgonians (Aguilar and Sanchez 2007; Grajales et al. 2007; Duenas and Sanchez 2009), but also in population genetic studies (Costantini et al. 2007).

2 THE TARGET SPECIES

2.1 Octocorallia

Octocorallia (also known as Alcyonaria) is a subclass of Anthozoa including 3 orders: Alcyonacea, Helioporacea, and Pennatulacea for a total of around 3,000 species with a worldwide distribution, occurring from litoral waters to deep-sea abyss (Weinberg 1976; Leen van Ofwegen el al. 1994; Gori et al. 2012). Octocorals are colonial organisms with a complex life cycle including a motile planktonic phase (larvae) and a later characteristic sessile phase (polyps). Most species have a calcium carbonate skeleton consisting of numerous sclerites, and many taxa present a calcified axis made of gorgonin. Polyps are embedded in the coenenchyme, which is penetrated by the solenia, a complex system of tubes that connected all the polyps in the colony. In the upper part of the polyps is located the mouth, provided by eight contractile tentacles, which are almost pinnate. The mouth extends into a gastrovascular cavity, with eight mesenteries that contain digestive gland cells, and in some species produce the gonads (Figure 2).

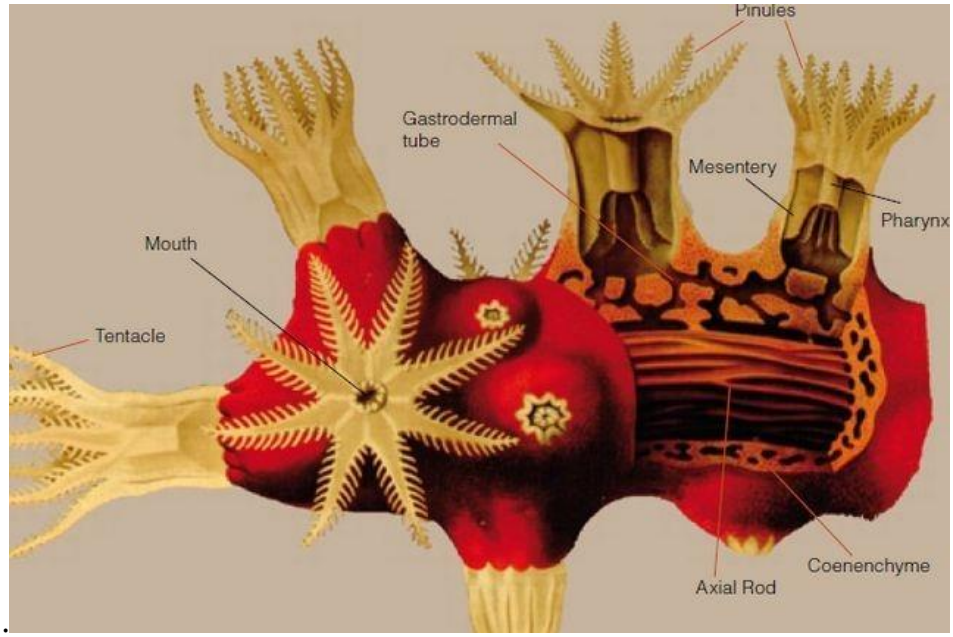

Figure 2. Scheme of the internal and external morphology of Octocorallia colony. (Leen van Ofwegen el al. 1994)

2.2 *Eunicella* genus

The genus *Eunicella* belongs to the family Gorgoniidae (Lamouroux 1812). It is characterized by the arrangement of the colonies, in a branch or stem-like shape with a central core. The morphology (i.e. shape, branching, and attachment method) of the colony and shape of the sclerites are used to distinguish between species (La Greca 1992). Currently, *Eunicella* genus includes about 35 species (Table 1) mainly distributed in shallow waters along the Eastern Atlantic and Mediterranean Sea, as well as a few deep-sea Atlantic and Pacific species (Grasshoff 1992). For example, *E. filiformis* (Studer 1879) and *E. verrucosa* (Pallas 1766) have mainly Atlantic distribution, being rarely reported in the Mediterranean Sea, either some patchy populations (*E. verrucosa*), or in the Straits of Gibraltar and the Alboran Sea area (*E. filiformis*) (Stiasny 1938; Rossi 1959; Carpine and Grasshoff 1975; Grasshoff 1992). Conversely, *E. cavolinii* (Koch 1887) and *E. singularis* (Esper 1791) are very common species in the Western Mediterranean Sea (Rossi 1959; Carpine and Grasshoff 1975).

EUNICELLA	SPECIES
Eunicella elba	*Eunicella labiata*
Eunicella albatrossi	*Eunicella lata*
Eunicella albicans	*Eunicella modesta*
Eunicella cavolini	*Eunicella multituberculata*
Eunicella ctenocelloides	*Eunicella palma*
Eunicella dawydoffi	*Eunicella papillifera*
Eunicella densa	*Eunicella papillosa*
Eunicella dubia	*Eunicella pendula*
Eunicella filifica	*Eunicella pergamentacea*
Eunicella filiformis	*Eunicella pillsbury*
Eunicella filum	*Eunicella pustulosa*
Eunicella furcata	*Eunicella racemosa*
Eunicella gazella	*Eunicella rigida*
Eunicella germaini	*Eunicella singularis*
Eunicella gracilis	*Eunicella stricta*
Eunicella granulata	*Eunicella tenuis*
Eunicella hendersoni	*Eunicella tricoronata*
Eunicella kochi	*Eunicella verrucosa*

Table 1. All the known species of *Eunicella.*

Eunicella species are sessile benthic suspension feeder and long-lived species, displaying slow growth rates and low natural mortality rates (Weinberg and Weinberg 1979; Mistri and Ceccherelli 1993; Bramanti et al. 2009). Their slow population dynamics make gorgonians especially vulnerable to direct and indirect anthropogenic disturbances (Garrabou and Harmelin 2002; Santangelo et al. 2007; Linares and Doak 2010) as anchors, fishing nets, and recreational divers (Bavestrello et al. 1997; Coma et al. 2004). In the last years several populations have suffered mass mortality events likely due to indirect effects of the increasing water temperatures (Coma et al. 2006; Linares et al. 2008; Garrabou et al. 2009). During two damage episodes at the end of the summers of 1999 and 2003 in the Northwestern Mediterranean (Cerrano et al. 2000; Perez et al. 2000; Garrabou et al. 2001; Fava et al. 2009) *E. singularis* was reported to be one of the most impacted species. From an ecological point of view, it

has a key role as engineer, providing biomass, structural complexity, refuge and habitat to a rich associated fauna in coralligenous and precoralligenous communities (Gili and Coma 1998; Ballesteros 2006).

2.2.1 *Eunicella cavolinii*

Eunicella cavolinii (Figure 3 and 4) is common in the eastern part of the western Mediterranean basin: French coast East of Marseilles, Italy, Adriatic, Eagean, Marmara (Carpine and Grasshoff 1975; Weinberg 1976). This species prefers vertical rocky walls on coralligenous formations in shallow coastal waters, often together with *Corallium rubrum* and *Paramuricea clavata* (Weinberg 1976) and overhanging rock ledges (von Koch 1887; Carpine 1963) but was also found in caves and in sediments (Riedl 1966). Its depth distribution ranges from 8 to 150 meters (Rossi 1959; Carpine and Grasshoff 1975). The morphology of the colonies is variable. Generally branches lie in one plane, but some times they showed a bush-like appearance, maybe due to the rough water conditions (Weinberg 1976). In fact, some authors sustained that water turbulence is an important factor in determining the shape of the colonies, while the light seems to be a limiting factor for their growth (Weinberg 1976; Abel 1959; Riedl 1966; Velimirov 1973). The branches present numerous short ramifications that tend to be curved and pointing in many directions. The diameter of the terminal branches vary from 1 to 3 mm and the polyps are mainly located on the opposite side of the branches. In fact the colonies of *E. cavolinii* are characterized by low height to width ratio, short and ramified branches (Carpine and Grasshoff 1975; Weinberg 1976). Overall colony shape resembles *Leptogorgia sarmentosa* or *E. verrucosa* rather than *E. singularis* (Rossi 1959; Weinbauer and Velimirov 1998). The color of the colonies varies from light ochre to warm yellow orange, that vanish when they are dried, is due to the living tissue and not to the spicules, which are colorless. Zooxanthellae are never encountered in this specie, and the polyps show a slightly opaque light-yellow color (Weinberg 1976).

Figure 3. *Eunicella cavolinii.*
Photo by: http://www.umema.it

Figure 4. Detail of the polyps of *Eunicella cavolinii*.
Photo by: http://www.naturamediterraneo.com

2.2.2 *Eunicella verrucosa*

Eunicella verrucosa (Figure 5 and 6) presents a mostly Atlantic distribution, from Great Britain to Angola (Carpine 1963, Carpine and Grasshoff 1975, Weinberg 1976) and it is frequently encountered near the Gibraltar Straits, where colonizes rocky beds at depths ranging from 2 to 60 meters (Lafargue 1969, Hiscock 2003). In the Mediterranean is reported in the Alboran Sea and along the Moroccan and Algerian coasts (Grasshoff 1992), but is found less frequently in the northwestern basin: in Italy and France (Provence and Côte d'Azur, western coast of Corsica) and along the Spanish coasts (Berdar and Cavallero 1980; Balduzzi et al. 1992; Cocito et al. 2002; Coppo et al. 2009). It is usually found on the hard substrates of the lower circalittoral up to the upper limit of the bathyal area from about 35 m to 200 m (Carpine and Grasshoff 1975, Grasshoff 1992) while its presence at shallower depths is rare (Rossi

1959, 1961, Cocito et al. 2002). The color of the colonies is very variable from white to rose or light orange. *E. verrucosa* presents a fan appearance and a general height of about 25-30 cm. The branches present numerous ramifications, similar to *E. cavolinii* and polyps are mainly located on all side of the branches. Calyces are very high and confer the typical warty appearance.

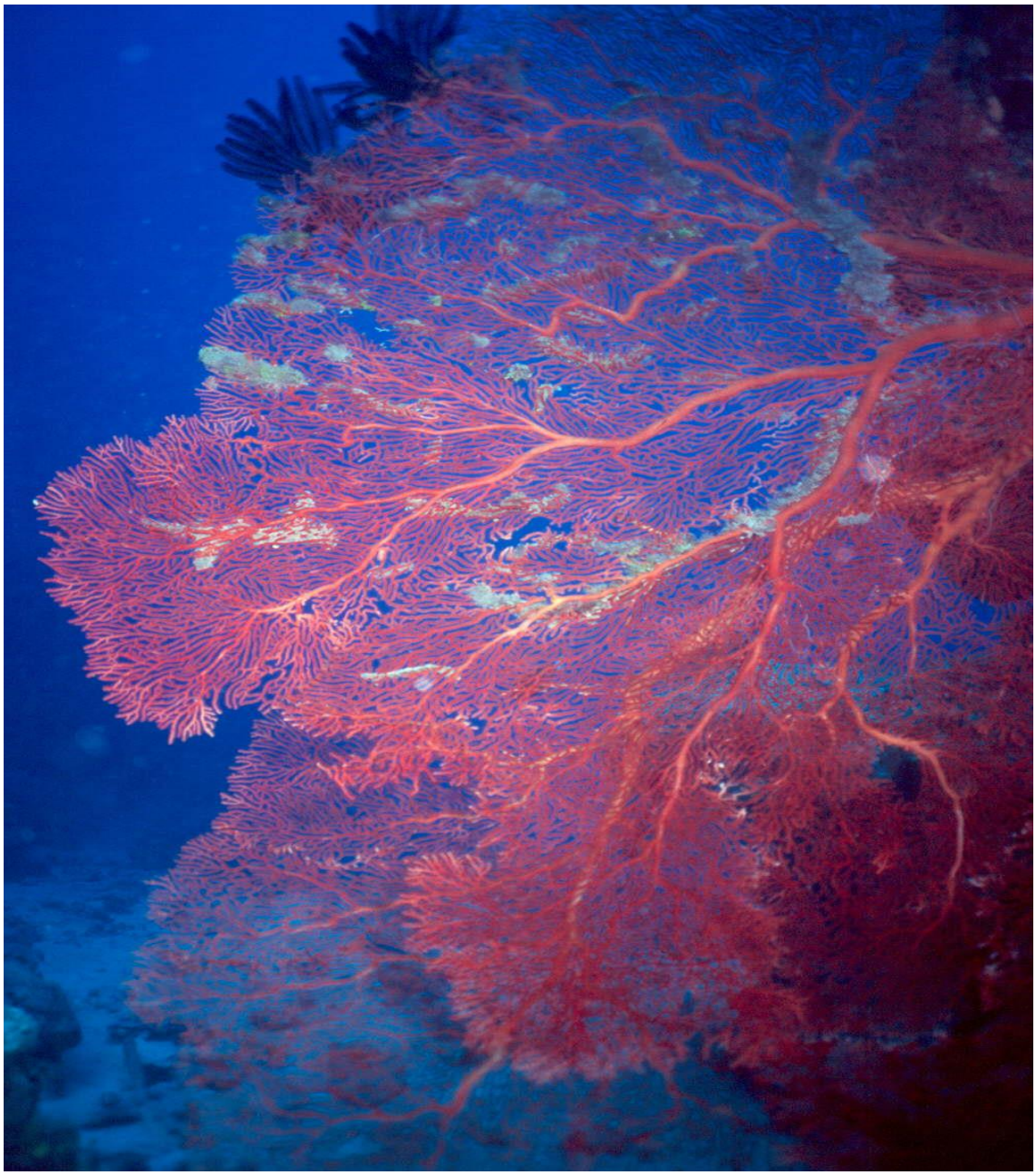

Figure 5. *Eunicella verrucosa*.
Photo by: http://blog.ocad.ca

Figure 6. Detail of the polyps of *Eunicella verrucosa*.
Photo by: http://www.marlin.ac.uk

2.2.3 *Eunicella racemosa*

No much information are available on this species. It was described by H Milne-Edwards, J Haime (1857). This is a very distinctive species, up to 2 meter high observed at 15 m depth (Lopez-Gonzeles P, pers. comm).

2.3 Eunicella singularis

Eunicella singularis is a long lived arborescent gorgonian (estimated life: 20-30 years). It is common in the western Mediterranean and occasionally in the eastern Mediterranean (Carpine and Grassoff 1975; Weinberg 1976; Gori et al. 2011) (Figure 7 and 8). It is abundant on shallow rocky bottoms and in deep sublittoral waters, form 10 to 220 meters (Rossi 1959; Théodor 1971). In 2013, dense populations of *E. singularis* have been reported at 100 m relying on information acquired through remotely operated vehicles (ROVs) (Rossi et al. 2008; Bo et al. 2009, 2011; Gori et al. 2011a). Water current seems to be a limiting factor for its distribution as the species seems to prefer localities with strong currents > 120m/H (Pax and Muller 1962; Weinberg 1965). The color of the colonies varies from bright white to dirty grey, the branches are relatively long and straight, parallel to each other, lie in one plane and present rather few ramifications. The diameter of the terminal branches varies from 2 to 3 mm; the polyps are present in all sides, protrude from lobate calyces and reach 3 mm length when are fully extended (Weinberg 1976). In some younger stages calyces are formed by eight lobes (Koch 1887), while in later stages are made by five smaller lobes and a big one (Weinberg 1976). The rind presents a granular appearance due to the outmost layer, which consists of balloon-clubs lying with their heads outwards while the deeper layer consists of spindles (Weinberg 1976). Moreover there are others spindles located in the anthocodia, the basal part of the tentacles (Théodor 1971). They are formed by flat, fairly smooth structures and their numbers vary greatly from 0 to 58 for polyps. The elastic properties of the axial skeleton allow colonies to bend and return to an erect position but sclerites limited this movement (Jeyasuria and Lewis 1987).

Figure 7. *Eunicella singularis.*
Photo by: http://www.webplongee.com

Figure 8. Detail of the polyps of *Eunicella singularis.*
Photo by: http://gps-tsc.upc.es

E. singularis is a gonochoric brooding species. Sexual reproduction occur annually in late May–June (Gori et al. 2007; Ribes et al. 2007), The importance of this species is due to its role as engineer species in coralligenous communities, to its aesthetic value and to the presence of symbiotic photosynthetic dinoflagellates commonly named zooxanthellae, largely assigned to the genus *Symbiodinium*, 'temperate A' or A' ribosomal haplotype (Bythell et al. 1997, Savage et al. 2002, Barbrook et al. 2006, Visram et al. 2006). Zooxanthellae fix carbon dioxide; produce oxygen and transfer photosynthates and oxygen to the host.

2.3.1 Shallow and deep morphotypes

Gori et al. (2012) evidenced the presence of two different *Eunicella* morphotypes, a shallow (Figure 9) and a deep one (Figure 10). They vary in color, colony shape and sclerite features. The principal discriminating characteristic is the presence/absence of symbiotic algae, indicating that *E. singularis* is not an obligatory symbiotic (Gori et al. 2012). The shallow morphotype inhabits horizontal and sub horizontal rocky bottoms at 20-30 meters, corresponding with the commonly known *Eunicella singularis*. Its colonies present a dirty grayish-white color due to the presence of symbiotic algae (Carpine and Grasshoff 1975; Weinberg 1976), a candelabra like-shape with few ramifications and large primary branches that may allow them to withstand strong water movements caused by storm-induced waves (Hiscock 1983; Bongaerts et al. 2010), large balloon club sclerites with smooth heads, wide collars and spiny ends, larger spindles in exposed compared with sheltered sites (Skoufas 2006). Furthermore they are characterized by small and no-reproductive colonies from 1 to 10 mm and a patchy distribution due to competition with fast growing algae and mortality induced by strong water movement causing colony detachment or toppling (Birkeland 1974; Yoshioka and Yoshioka 1991; Weinbauer and Velimirov 1996b; Gori et al. 2012).

Figure 9. Shallow *Eunicella singularis* morphotype.
Photo by: http://farm4.static.flickr.com

The deep morphotype covers rocky sublittorals at 40–60 m and lacks symbiotic algae. Its colonies show a bright white color due to lack of zooxanthellae (Théodor 1969; Weinberg 1976) and present more variable colony shape. They have more ramifications, shorter primary branches; and balloon club sclerites varying with increasing depth (Gori et al. 2012). These deep populations are composed by larger reproductive colonies (Gori et al. 2011b), the height ranged between 10-20 cm and have more uniform spatial distribution. The larger size and distribution can be related to the decrease with depth of water movement induced by waves action and the absence of fast growing algae (Grigg et al. 1977; Lasker et al. 1991;Gori et al. 2012).

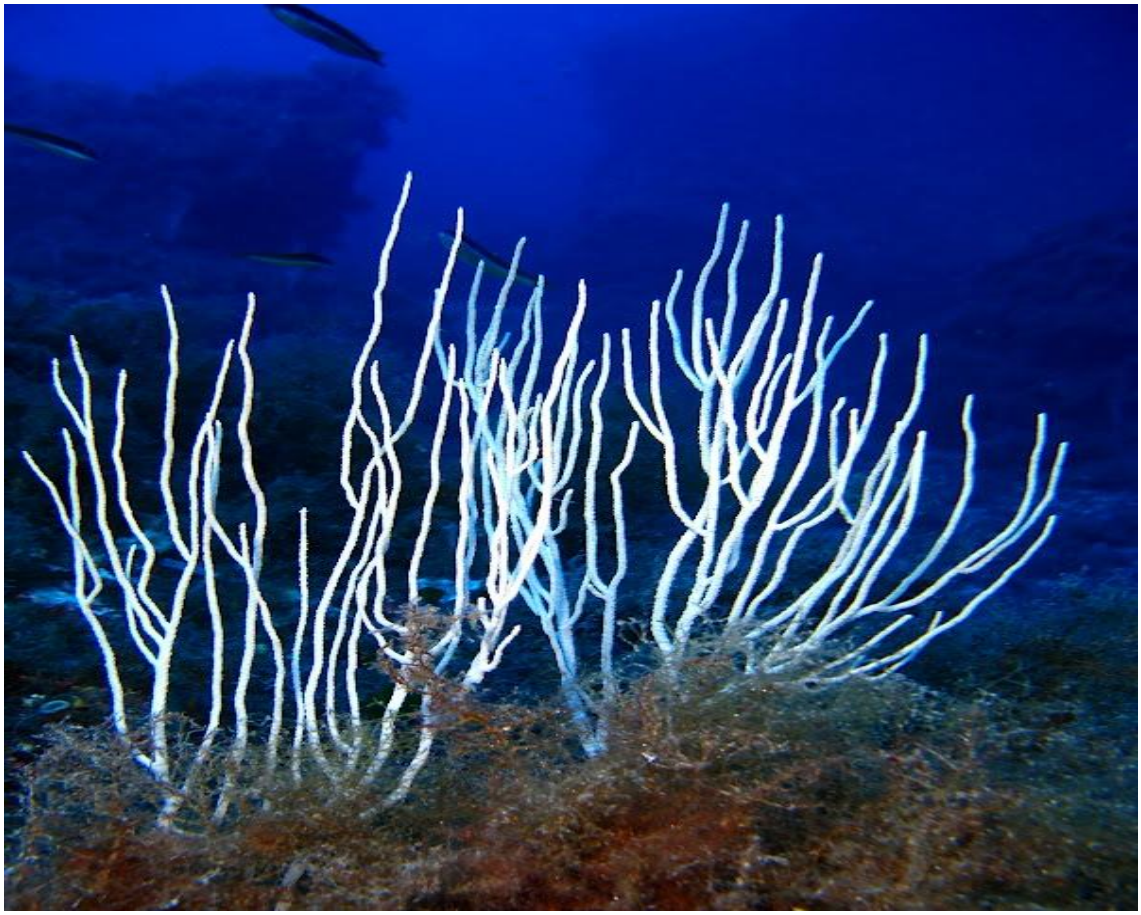

Figure 10. Deep *Eunicella singularis* morphotype.
Photo by: http://1.bp.blogspot.com

The two morphotypes partially overlap at around 30-40 meters depth that correspond to the summer thermocline which could acts as a physical barrier (Coma et al. 2000; Gori et al. 2011). Some authors consider the symbiotic and aposymbiotic colonies as intraspecific varieties (Carpine and Grasshoff 1975) while some others recognize two different subspecies (Weinberg 1976). For example, this deep morphotype was first described by Théodor (1969) and was proposed as a subspecies (*Eunicella stricta aphyta*).

2.3.2 Eunicella stricta aphyta

Théodor (1969) described a deep morphotype as a subspecies of *E. singularis* and proposed it as "*Eunicella stricta aphyta*". *Eunicella stricta aphyta* presents a rose

salmon color, inhabits deeper waters down to 220 m in Cap de Creus, and lacks symbiotic algae. Moreover, was reported that *E. stricta aphyta* presents a colony shape, spicules and sclerite dimension very similar to exposed colonies of *E. cavolinii* (Gori et al. 2012). In fact, Bo et al. (2011) define this deep morphotype as *Eunicella cavolinii.*

2.3.3 Previous genetic studies

As for other Gorgonacea (Shearer et al. 2002; Calderon et al. 2006; Costantini et al. 2013) also in *Eunicella* mitochondrial DNA have shown very low rate of variability, maybe due to a slow accumulation of mutations or a higher efficiency of repair systems. In fact, Calderon et al. (2006) analyzing different populations of *E. singularis* and *E. cavolinii* showed that all the individuals shared the same COI sequences regardless their geographic origins. The mutS homolog gene (*msh1*) sequences were used to understand phylogenetic position of the two morphotypes of *E. singularis,* and *E. cavolinii,* and *E. verrucosa.* Based on the presence of only a single *msh1* haplotype shared among all the analyzed colonies, Gori (2012) suggested that this marker was not useful to discriminate between the two morphotypes of *E. singularis* and among the different species of *Eunicella.* Moreover, all these results could also suggested that effectively the two morphotypes are a consequence of phenotypic variation within a single species of *Eunicella* or that the morphotypes could be affected by a recent speciation event that is not yet evident with these mitochondrial markers (Gori et al. 2012). Abdoullaye et al. (2010) developed species specific microsatellite loci for *Eunicella singularis*. These loci were used by Pey et al. (2013) to explore the genetic differentiation between populations of *E. singularis* at different spatial scales: 1) two depths: 10 to 15 m and 35 to 40 m; 2) two different sites: Medes (Spain) and Riou Islands (France). No significant differences between "shallow" and "deep", but significant differences were observed between the two sites (Pey et al. 2013). The Internal Transcribed Spacer 2 (mitochondrial ITS 2) has been used to determine the genetic structure of *E. cavolinii* populations (Calderon et al. 2006). This study showed a high intra-specific level of variability. In fact, observed intra-individual variability was of the same order of the inter population variability. Such a great variability of

ITS2 sequences in *E. cavolinii* makes, at this moment, this molecular marker an unhelpful resource for this species. Conversely, ITS1 has been used to define the genetic structuring of another octocoral species with interesting results (Costantini et al. 2013). Based on the fact that few molecular markers are actually useful for gorgonians population genetic studies, in this study ITS-1 sequence polymorphism and microsatellite loci have been used.

3 AIMS

Despite their importance as engineered species in the Mediterranean Sea, *Eunicella* taxonomy, systematics, and biogeography remain poorly understood. In particular, *Eunicella singularis* showed two depth-related morphotypes for which the taxonomic status is not yet clarified. To increase these knowledge two different studies were carried out. Firstly, we tried to elucidate whether the two *E. singularis* morphotypes are genetically isolated. To address this goal 6 populations were sampled in Cap de Creus (Spain) at ten meters distance along a vertical gradient from 10 to 60 meters depth. Microsatellites and ITS1 polymorphism sequences were used to test whether genetic variability and/or structuring vary with depth.

Moreover, a preliminary work was carried out to evaluate the phylogenetic relationship among species of the genus *Eunicella*. In particular, the aims were:

1) to identify a suitable molecular marker able to distinguish among these species
2) to clarify the phylogenetic relationship among the species of the genus *Eunicella* and the systematic position of the two morphotypes.

4 MATERIALS AND METHODS

4.1 Site description and experimental design

The site chosen was on the northern coast of the Cap de Creus (42°18'44'' N; 03°19'05'' E), in the northwestern Mediterranean Sea (Figure 11). This is a metamorphic rocky coast, showing an alternation of vertical cliffs with smooth ramps. The general circulation pattern is dominated by the Northern current (Millot 1990) and is directly exposed to a strong near-bottom current coming from the north (DeGeest et al. 2008), as well as to the main winds and wave action. Six populations of *Eunicella singularis* were collected along a vertical gradient from 10 to 60 meters depth. Each population was 10 meter distant from the other. In each population, from 16 to 19 colony fragments were collected.

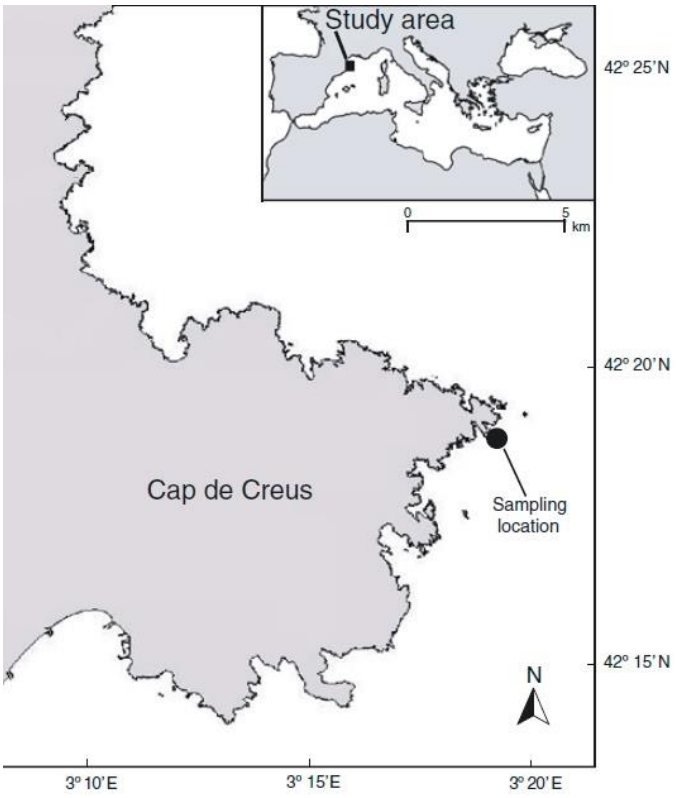

Figure 11. Map of the study area. The position of the sampling location along the Cap de Creus coast is indicated.

Then, different species of *Eunicella* were sampled.

- 5 colonies of *E. cavolinii* were collected by Lorenzo Bramanti at Elba Island (Italy) between 18 and 20 meters
- 5 colonies of *E. verrucosa* were collected by Gori Andrea at Terragona (Spain) between 13 and 17 meters
- 16 colonies of *Eunicella sp.* (*Eunicella stricta aphyta*) were collected by Gori Andrea at the continental shelf of Cap de Creus (Spain) at around 100 meters depth by means of a Remotely Operate Vehicle (ROV)
- 6 colonies of *E. racemosa* were collected by Pablo Lopez Gonzalez (University of Sevilla) along the Morocco coast (Atlantic Ocean) at 15 meters depth.

All colonies were stored in 80% ethanol.

4.2 DNA extraction

Total genomic DNA was extracted from three to five polyps per colony fragments following the cetyl trimethyl ammonium bromide (CTAB) procedure (Miller et al., 1987):

- Polyps dried put into 1.5mL tubes;
- Add 400 µl of 2X CTAB [1,4 µL NaCl; 20mM EDTA; 100mM Tris; pH 8.0; 2% CTAB; 0.2% 2-mercaptoetanolo];
- Proteolysis by the use of Proteinasi K (4µL of water 20mg/mL);
- Put at 43°C for overnight;
- Remove protein residual by the use of 1 volume of a mix 1:1 of Chloroform;
- Mix for 5 minutes at 12000 rpm;
- Take the upper part and put in a sterile tube;
- Put 400 µL of EtOH 100% to precipitate nucleic acid;
- Keep refrigerate for one hour at 4°C;
- Centrifuge for 15 minutes at 4°C and 14000rpm;
- Throw the content and DNA will remain into the walls of tubes;
- Put inside 150 µL of EtOH 70% and centrifuge for 5 minutes at 4°C and 14000 rpm;
- Remove upper part and dry at 37°C for 20 minutes;
- Put 20 µL of TE1X (Tris 0.1M; EDTA 0.01 M; pH 8) to suspend DNA

4.3 Microsatellite loci

4.3.1 Genotyping and binning

After DNA extraction all the individuals were genotyped using 5 microsatellite loci developed by Abdullaye et al. (2010) (Table 2) and following their protocols.

Locus name	Primer sequence (5'-3')	Label	Core
Caes 64	F CTGGCAAAGCTTCAGTCTCG R ACTGGCCAAGTGCAGTATCC	ROX	(GT)13
C 40	F AGATGAGAAAGGACGGTCGG R CACCAGCAACAACAACTGC	HEX	(GTT/GCT)24 *interrupted*
S 10	F AGTTCACACGAAGTATTGGGC R TCGTCATTTCGGCTCGTTC	HEX	(ATT)7
C30	F TCGCCCGAGTTGTTGAGGC R ACGTAGCACCATAGTACCG	TAMRA	(ATGT)5
C21	F TGGGATGTCAAGTGGTTTTCAAG R CCGGTTTCAGGTTCATGCC	FAM	(AAT)10

Table 2. SSR loci from Abdullaye et al. (2010) used in this study: primer sequences, fluorochrom, and microsatellite core.

Loci CAES-64, S10, C30, C40 were amplified in multiplex using a QIAGEN® Multiplex PCR Kit using polymerase chain reaction (PCR) conditions (Table 3 and 4).

Reagents and conditions for Multiplex amplification	
Mix	6.25 µL
Q	1.25 µL
Water	1.25 µL
Primer Mix	1.25 µL
DNA	2.5 µL

Table 3. Reagents and their quantity to perform PCR in Multiplex.

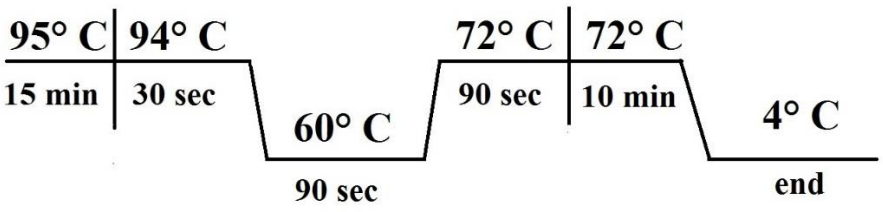

Table 4. Cycle used for the multiplex PCR.

Locus C21 was amplified following protocol and cycle condition as in Table 5 and Table 6.

Reagents and cycle conditions	C 21	
Mg 2+	1.2	µL
Buffer	2	µL
dNTP	1	µL
Taq	0.2	µL
Primers F,R	1	µL
Water	12.6	µL
DNA	1	µL
Annealing T°	55° C	
Cycles	35	

Table 5. PCR condition used for the amplification of the C21 locus.

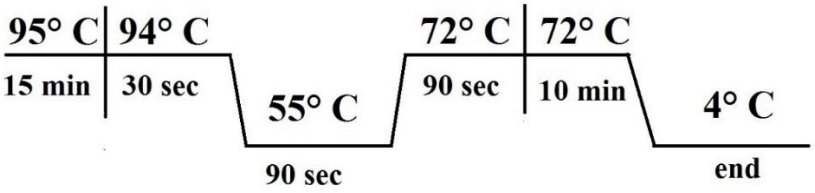

Table 6. Cycle used for the amplification of the C21 locus.

Genotyping of individuals was carried out on an ABI 310 Genetic Analyser (Applied Biosystems), using forward primers labeled with FAM, HEX, TAMRA, ROX (Sigma) and LIZ HD500 (Applied Biosystems) as internal size standard through MACROGEN INC. Service. Allele sizing was determined using Peak Scanner v1.0 software from Applied Biosystems, Inc. This software provided allele size values as numbers with two decimals. Software for the analysis of microsatellite needed integer values; it is necessary to assign an integer (bin) to the scored alleles according to their length in base pairs, so the raw values had to be trundled off (binning). This analysis was done using a MS Excel worksheet. Raw data with decimals were examined locus by locus, to determine if the raw allele size distribution respects the distribution according to the core repeat and to define the allele range for each locus to round each allele according

to the defined range This technique can introduce errors in the allele frequencies but it's very useful to find out mistakes committed during the first phase of scoring. The allelic range for each locus was then compared with that define by Abdullaye et al. (2010) to double check the conformity of the binning.

4.3.2 Microsatellite genetic variability and structuring

Linkage disequilibrium analysis for pairs of loci was based on the likelihood ratio test with the EM algorithm (Dempster et al. 1977) through 10,000 permutation procedures (number of initial conditions for EM, 100) using ARLEQUIN 3.5 (Excoffier and Lischer 2010). Individuals sharing the same multilocus genotype (MLG) were checked using GENALEX version 6.1 (Peakall & Smouse 2006). Identical MLGs can be the result of two different genotypes originated by two distinct sexual reproduction events but sharing the same alleles for all genotyped loci. To test for this, the unbiased probability of identity (PID) (Kendall and Stewart 1977) that two sampled individuals share identical MLG by chance, through sexual reproduction was computed. All the analyses were performed using the entire data set and the data set corrected using only single MLG. Genetic diversity within populations for each locus and over all loci was estimated as observed (Ho) and expected (Hs) heterozygosity using the GENETIX software package Version 4.05 (Belkhir et al. 2004). Single- and multilocus F_{IS} values were estimated using Weir and Cockerham's f (Weir & Cockerham 1984), and significant departures from the Hardy–Weinberg equilibrium were tested using "Fisher's exact test" in GENEPOP version 3.4 (Raymond & Rousset 1995) as implemented for online use (http://genepop.curtin.edu.au/), with the level of significance determinate by a Markov-chain randomization. For this analysis 1000 steps of dememorization, 100 batches and 1000 iterations per batch, with the aim to obtain a significance level of 1% were selected. Significant differences in terms of genetic diversity (e.g. allelic richness, Ho, Hs), between populations above 30 meter depth and those below this depth were tested using a permutation procedure (1000 iterations) in FSTAT version 2.9.3.2 (Goudet 2001).

Te genetic divergence among populations was determined using Weir & Cockeram's (1984) F_{ST} estimator in the ARLEQUIN Version 2.0 software (Schneider et al. 2000).

Genotypic differentiation among populations was tested with an exact test (Markov chain parameters: 1000 dememorizations, followed by 1000 batches of 1000 iterations per batch). Non-metric multidimensional scaling analysis (MDS) implemented by PRIMER v6 (Clarke & Gorley 2006) was used to represent the results. The partition of genetic variance among populations was conducted through analysis of molecular variance (AMOVA) implemented in ARLEQUIN. The AMOVA 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. Groups were defined based on the results of the pairwise F_{ST}. Pairwise F_{ST} estimates were used to calculate the relationship between genetic differentiation and geographical distance by regressing $F_{ST}/(1 - F_{ST})$ values against the natural logarithm of the distance between population pairs using the Mantel test (Mantel 1967) with 10,000 permutations, as implemented in GENEPOP. The number of clusters detected among tiles, K, was estimated by employing a Bayesian approach implemented in the program STRUCTURE version 2.2 (Pritchard et al. 2000; Falush et al. 2003, 2007). Each individual was assigned to the more probable common clusters based on the similarity of their multilocus genotypes at six microsatellite loci. Mean and variance of log likelihoods of the number of clusters for K = 1 to K = 10 were inferred by running structure five times with 500 000 repetitions each (burn-in = 50 000 iterations) under the admixture ancestry model and the assumption of correlated allele frequencies among samples as suggested in Falush et al. (2003). Following the recommendations of Evanno et al. (2005), the ad hoc statistic ΔK based on the rate of change in the log likelihood of data between consecutive K values were calculated through the web based program STRUCTURE HARVESTER (available at http://taylor0.biology.ucla.edu/structureHarvester/). A discriminating analysis of principal components (DAPC) as implemented in the ADEGENET software, version 1.3 (Jombart et al. 2010) was performed. This technique extracts information from genetic datasets (multivariate in nature) by first performing a principal component analysis (PCA) on pre-defined groups or populations, and then using the PCA factors as variables for a discriminating analysis (DA), which seeks to maximize the intergroup component of variation. The optimal number of clusters (populations) was

predicted using the k-means clustering algorithm, find clusters, retaining all principal components. In all analyses 10 principal components of PCA were retained as input to DA because little information is gained by adding PCs after the first 10. Significance levels for multiple comparisons of loci across samples were adjusted using a false discovery rate (FDR) correction for multiple tests (Benjamini and Hochberg 1995).

4.4 ITS-1

ITS1 sequences were amplified using the protocols reported in Table 7 and Table 8.

Reagents and cycle conditions	ITS1 Sequences
Mg 2+	2 µL
Buffer	2.5 µL
dNTP	1 µL
Taq	0.2 µL
Primers F,R	1.25 µL
Water	14.3 µL
DNA	2.5 µL
Annealing T°	60° C
Cycles	30

Table 7. PCR reagents used for the amplification of the ITS1 region.

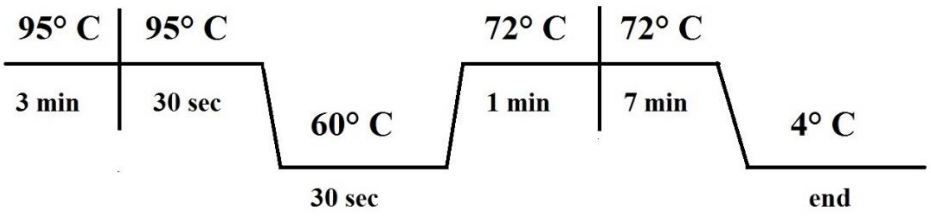

Table 8. Cycle used the amplification of the ITS1 region.

4.4.1. ITS-1 genetic variability and structuring

ITS1 sequences were analyzed using a biological sequences alignment editor Bio Edit (Ibis bioscience) version 7.2.5. Since some sequences contained several heterozygous sites, ITS sequence types were estimated using PHASE 2.1 (Stephens et al. 2001) on DNASP v.5 (Librado & Rozas 2009), which implements a coalescent-based Bayesian method to infer them. Number of haplotypes (h), haplotype and nucleotide diversity (Hd and π, respectively), were calculated DNASP 5.10.1 (Rozas and Rozas 1995). To perform a preliminary phylogenetic analysis of the genus *Eunicella* a dataset with all the individuals from the different species were created. Distance matrixes of sequence divergence among the species were calculated as pdistance (D*p*) in MEGAv.5.05. The best-fit substitution model for the dataset was calculated using JMODELTESTv.1.1 (Posada 2008) Mac software, considering 88 substitution models by hLRT calculator with 4-gamma category. Maximum likelihood analyses were performed using on Beast v. 1.7.5 using 15 million chains, 1 relaxed clock lognormal. The first 1000 tree were buring using TREE ANNOTATOR, and the phylogenetic tree was visualized as posterior probability using FIGTREE v. 1.4.0. ITS sequence of *Leptogorgia sarmentosa* obtained by Poliseno A. (2007) were used as outgroup.

5 RESULTS

5.1 Depth gradient according to microsatellite loci

99 individuals were genotyped for the 5 loci. No null alleles were observed. Tests of linkage disequilibrium between loci within populations indicated no significant association of alleles (all $p > 0.05$), confirming that all loci are independent markers. 61 Multi Locus Genotypes (MLGs) were identified, among which 12 were shared by two or more colonies (Tables 9 and 10).

A	285285196196191191195198181181g
B	285291196196191191189189181181g
C	285291196196191191189195181181g
D	285291196196191191195195181181g
E	285291196196191191195198181181g
F	285291196196191191198198181181g
G	291291190190199199195195181181g
H	291291196196191191195195181181g
I	291291196196191191195198181181g
J	291291196196191191198198181181g
K	291291196196199199195195181181g
L	291291196196199199198198181181g

Table 9. Shared Multilocus Genotypes found in the dataset.

	Genotypes											
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**
Populations												
10 m	0	0	0	0	0	0	0	0	**1**	**3**	0	**1**
20 m	0	0	0	0	0	0	0	0	0	0	0	**1**
30 m	0	0	0	0	0	**2**	**2**	**1**	0	**1**	0	**2**
40 m	**1**	**10**	**5**	**1**	**1**	0	0	0	0	0	0	0
50 m	0	0	**0**	**1**	0	0	0	**1**	0	0	**9**	0
60 m	**1**	0	**2**	**1**	**2**	0	0	**0**	0	0	0	0

Table 10. Distribution of the shared MLGs among all the populations.

The probability that each of these genotypes was produced through sexual reproduction was high (PID =0.058).

The following analyses were performed using first the entire data set, and afterwards including only one individual from those that shared the same MLGs.

5.1.1 Complete dataset

The number of alleles per locus ranged from 1 (in S10, C30 and CAES-64) to 5 (C21). Expected heterozygosity (Hs) ranged between 0.00 (in S10, C30 and CAES-64) and 0.74 (C21); and observed heterozygosity (Ho) ranged between 0.00 (in S10, C30, C21 and CAES-64) and 0.89 (in C40). Estimates of multilocus expected heterozygosity (Hs) ranged between 0.18, in the ES_40 m population, and 0.45 in the ES_20 m population; and observed heterozygosity (Ho) ranged between 0.05 in the ES_50 m population and 0.31 in the ES_20 m population. Significant deviations from HW equilibrium were observed in all populations but not at 60 meter depth. Three populations showed a deficit of heterozygotes with positive values of F_{IS}, while two populations (ES_40m and ES_60m) showed an excess of heterozygotes with negative F_{IS} values (Table 11).

Locus	Sample (n)					
	ES_10m (16)	ES_20m (16)	ES_30m (16)	ES_40m (19)	ES_50m (16)	ES_60m (16)
C40						
Na	3	3	2	2	2	2
Ho	0.25	0.37	0.25	0.89	0.06	0.62
Hs	0.40	0.40	0.38	0.49	0.06	0.47
Fis	0.41 *	0.09	0.36	-0.80 **	0.00	-0.30
S10						
Na	3	2	3	1	1	2
Ho	0.18	0.18	0.12	0.00	0.00	0.25
Hs	0.43	0.48	0.49	0.00	0.00	0.21
Fis	0.59 **	0.63 *	0.75 **	------	------	-0.11
C30						
Na	3	3	3	1	2	2
Ho	0.25	0.43	0.06	0.00	0.12	0.06
Hs	0.49	0.42	0.54	0.00	0.37	0.06
Fis	0.52 **	-0.01	0.89	------	0.68 *	0.00
C21						
Na	5	5	3	3	3	4
Ho	0.37	0.56	0.00	0.36	0.06	0.56
Hs	0.59	0.74	0.53	0.43	0.22	0.45
Fis	0.39 **	0.27 **	1.00 **	0.18	0.73 **	-0.21
CAES64						
Na	1	2	1	1	2	2
Ho	0.00	0.00	0.00	0.00	0.00	0.00
Hs	0.00	0.22	0.00	0.00	0.30	0.22
Fis	------	1.00 **	------	------	1.00 **	1.00 **
MULTILOCUS						
Na	3	3	2.4	1.6	2	2.4
Ho	0.21	0.31	0.08	0.25	0.05	0.3
Hs	0.38	0.45	0.38	0.18	0.19	0.28
Fis	0.47*	0.33 *	0.78 **	-0.33 *	0.75 *	-0.02

Table 11. Genetic diversity within population at 5 microsatellite loci; n: total number of individuals genotyped; Na: number of alleles per locus computed over all loci; Ho: observed heterozygosity, Hs: gene diversity (Nei 1987), F_{IS} Weir and Cockerham's (1984) estimate fixation index. Asterisks types indicate significant deviations from HWE after FDR.
** P <=0.01; * P <0.05.

Significant differences were observed between shallow (above 30 m depth) and deep populations (below 30 m depth) in terms of Hs (0.01) and allelic richness (0.03) while Ho is not significant (0.51). Pairwise multilocus estimates of F_{ST} ranged from 0.02 (between ES_30m and ES_20m) to 0.56 (between ES_40m and ES_50m). Pairwise estimates were not significant between ES_10m, ES_20m and ES_30m, while they were significant for all the other comparisons (Table 12). MDS in Figure 12 gives a graphic representation of the pairwise F_{ST} estimates.

	10 m	20 m	30 m	40 m	50 m	60 m
10 m	0.00					
20 m	0.08	0.00				
30 m	0.03	0.02	0.00			
40 m	**0.31**	**0.40**	**0.39**	0.00		
50 m	**0.33**	**0.23**	**0.26**	**0.56**	0.00	
60 m	**0.25**	**0.33**	**0.29**	**0.19**	**0.41**	0.00

Table 12 . Pairwise multilocus estimates of F_{ST} (Weir & Cockerham 1984) between all the *Eunicella singularis* populations. Bold numbers are statistically significant at P=0.01 after false discovery rate correction.

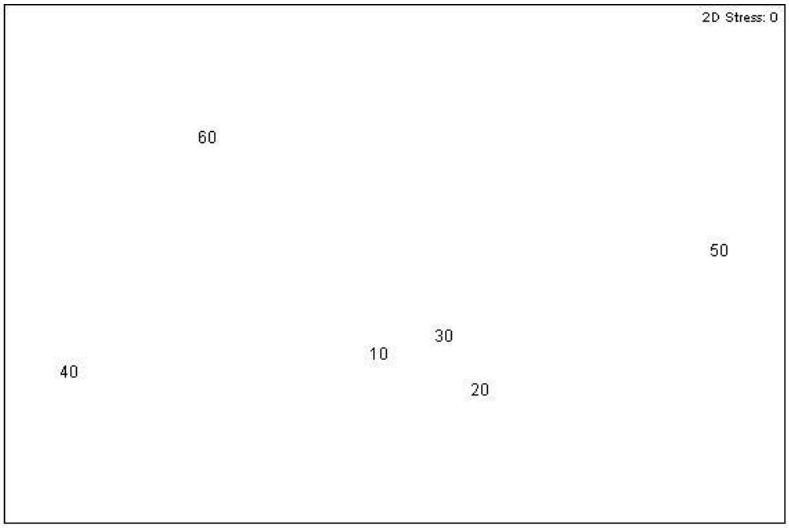

Figure 12. MDS of the pairwise F_{ST} estimates.

The AMOVA revealed that the 68.5% (p <0.001) of the total genetic variation occurred within populations. The percentage of the variation among populations within groups was also significant (19.85% P<0.01), while among groups the percentage of variation was low and not significant (Table 13).

Source of variation	d.f	Sum of squares	Variance components	Significance tests	Percentage of variation
Among groups	1	22.72	0.13	0.72 +- 0.008	11.59
Among populations within groups	5	40.65	0.22	0.00 +- 0.00	19.85
Within populations	223	172.46	0.77	0.00 +- 0.00	68.56
Total	229	235.84	112.804		

Table 13. Analysis of molecular variance (AMOVA) based on two groups: above and below 30 meter depth.

The relationship between genetic differentiation and geographical distance based on the Mantel test was not statistically significant (P= 0.43) (Figure 13).

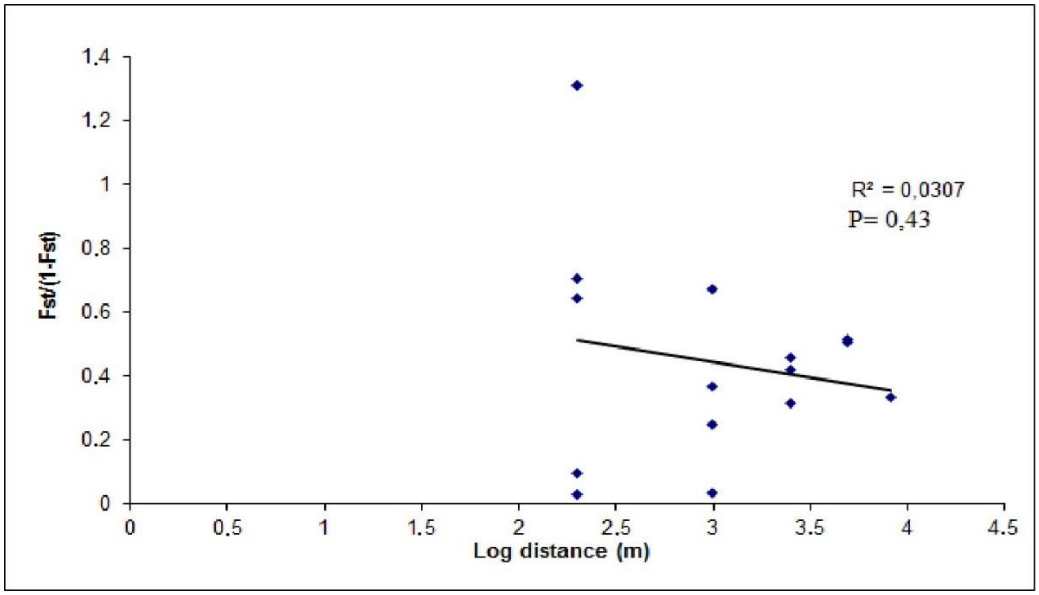

Figure 13. Relationship between genetic differentiation estimates and the logarithm of geographical distance between the *Eunicella singularis* populations.

The stepwise clustering procedure performed in STRUCTURE resulted in a separation of the populations into two clusters (following Evanno: K=2, ΔK=407.39). The first cluster is mainly composed by the individuls of the shallow populations (ES_10m, ES_20 m and ES_ 30m) and the ES_50m, while the second cluster ismainly composed by individulas belonging to the ES_40m and ES_60m populations (Figure 14).

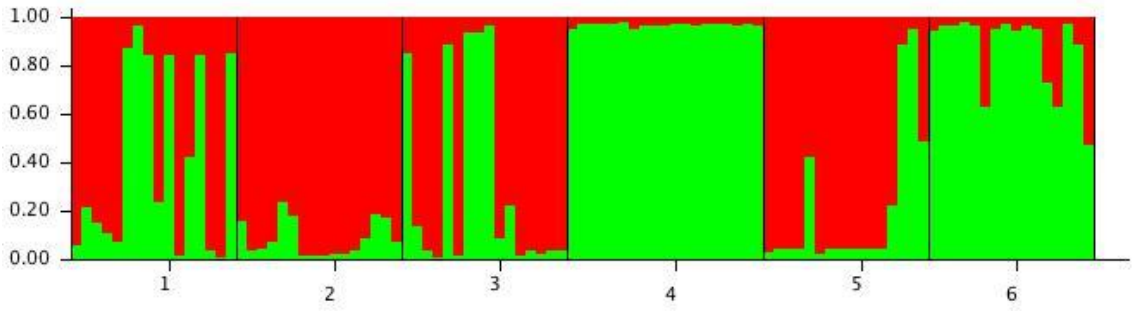

Figure 14. Results of the clustering analysis conducted in STRUCTURE. In the bar plot, each of the 99 individuals is represented by a vertical bar indicating its estimated proportion of membership to each cluster (represented by different colours). Numbers from 1 to 6 represent the 6 popoulations.

The DAPC procedure indicated that the 10 principal components of the retained PCA explained 95 % of the total variance. The scatter plot of the first two components of the DA showed similar results to the STRUCTURE asignment but with a greater distinctiveness of ES_50m with the other populations (Figure 15).

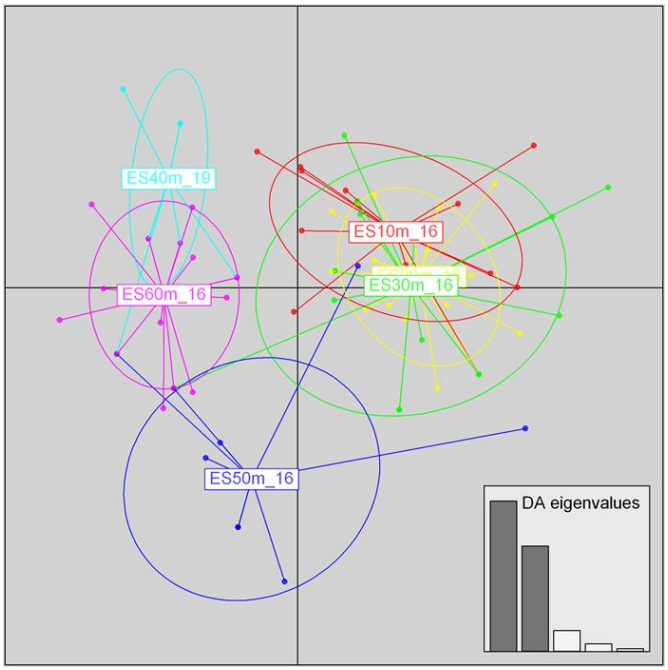

Figure 15. Plots of the first two axes obtained in the Discriminant Analysis of Principal Components using microsatellite dataset. Dots represent individuals.

5.1.2 Reduced Microsatellites dataset

The number of alleles per locus ranged from 1 (in S10, C30 and CAES-64) to 5 (C21). Expected heterozygosity (Hs) ranged between 0.00 (in S10, C30 and CAES-64) and 0.74 (C21); and observed heterozygosity (Ho) ranged between 0.00 (in S10, C30, C21 and CAES-64) and 0.66 (in C40). Estimates of multilocus expected heterozygosity (Hs) ranged between 0.21 in the ES_40 m population and 0.45 in the ES_20 m population; and observed heterozygosity (Ho) ranged between 0.09 in the ES_30 m population and 0.31 in the ES_20 m population (Table 14). Since the higher number of individuals that shared MLGs were observed in the ES_40m and ES_60 m populations, those were the populations that showed the larger differences compared to the analysis performed with the complete dataset. In fact, with the reduction of repeated MLG these two populations were in HW equilibrium.

	ES_10m (14)	ES_20m (16)	ES_30m (13)	ES_40m (6)	ES_50m (9)	ES_60m (14)
C40						
Na	3	3	2	2	2	2
Ho	0.28	0.37	0.23	0.66	0.11	0.57
Hs	0.44	0.39	0.39	0.44	0.10	0.45
Fis	0.39	0.09	0.44	-0.42	0.00	-0.20
S10						
Na	3	2	3	1	1	2
Ho	0.21	0.18	0.15	0.00	0.00	0.28
Hs	0.48	0.48	0.50	0.00	0.00	0.24
Fis	0.58 **	0.63 *	0.71 **	------	------	-0.13
C30						
Na	3	3	3	1	2	2
Ho	0.28	0.43	0.07	0.00	0.22	0.07
Hs	0.53	0.41	0.56	0.00	0.49	0.06
Fis	0.49	-0.01	0.87 **	------	0.59	-0.00
C21						
Na	5	5	3	3	3	4
Ho	0.42	0.56	0.00	0.50	0.11	0.50
Hs	0.63	0.74	0.54	0.62	0.36	0.41
Fis	0.35 *	0.27 **	1.00 **	0.28	0.72	-0.17
CAES64						
Na	1	2	1	1	2	2
Ho	0.00	0.00	0.00	0.00	0.00	0.00
Hs	0.00	0.21	0.00	0.00	0.44	0.24
Fis	------	1.00 **	------	------	1.00 **	1.00 **
MULTILOCUS						
Na	3	3	2.4	1.6	2	2.4
Ho	0.24	0.31	0.09	0.23	0.08	0.28
Hs	0.41	0.45	0.4	0.21	0.28	0.28
Fis	0.45 *	0.33 **	0.78 **	0.00	0.71 **	0.03

Table 14. Genetic diversity within population at 5 microsatellite loci ; n: total number of individuals genotyped; Na: number of alleles per locus computed over all loci; Ho: observed heterozygosity, Hs: gene diversity (Nei 1987),F_{IS} Weir and Cockerham's (1984) estimate fixation index. Asterisks types indicate significant deviations from HWE.
** P <=0.01; * P <0.05.

Significant differences were observed between shallow (above 30 m depth) and deep populations (below 30 m depth) in terms of allelic richness (P=0.043) while Ho and Hs (P=0.04) were not significantly different (P=0.45). The main difference observed with the analysis on the complete dataset in terms of pairwise multilocus estimates of $F_{ST,}$ was the lack of significant differentiation between ES_40m and Es_60m (Table 15). Moreover, multilocus estimates of F_{ST} were low in the pairwise comparisons of shallow water populations. MDS in Figure 16 gives a graphic representation of the pairwise F_{ST} estimates.

	10 m	20 m	30 m	40 m	50 m	60 m
10 m	0.00					
20 m	0.006	0.00				
30 m	-0.04	-0.02	0.00			
40 m	**0.20**	**0.31**	**0.19**	0.00		
50 m	**0.24**	**0.22**	**0.24**	**0.44**	0.00	
60 m	**0.23**	**0.31**	**0.21**	0.07	**0.36**	0.00

Table 15. Pairwise multilocus estimates of F_{ST} (Weir & Cockerham 1984) between all the *Eunicella singularis* populations. Bold numbers are statistically significant at 0.01 after false discovery rate correction.

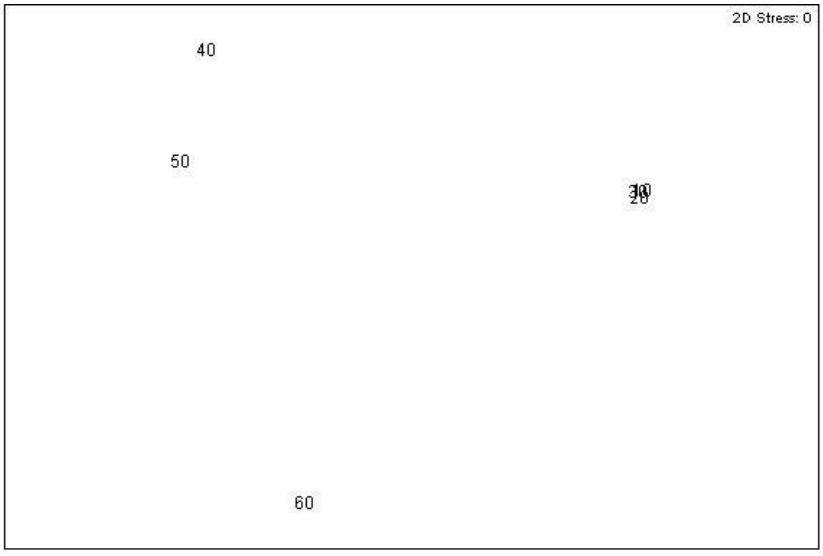

Figure 16. MDS of the pairwise F_{ST} estimates.

The AMOVA revealed that the 75.33 % (p <0.001) of the total genetic variation occurred within populations. The percentage of the variation among populations within groups resulted also significant (12.08 %, P<0.01) (Table 16).

Source of variation	d.f	Sum of squares	Variance components	Significance tests	Percentage of variation
Among groups	1	10.16	0.16	0.10557+-0.00992	12.58
Among populations within groups	4	12.64	0.15	0.00489+-0.00203	12.08
Within populations	80	77.22	0.96	0.00 +- 0.00	75.33
Total	85	100.02	1.28		

Table 16. Analysis of molecular variance (AMOVA) based on two groups: above and below 30 meter depth.

The relationship between genetic differentiation and geographical distance based on the Mantel test was not statistical significant (P= 0.76) (Figure 17).

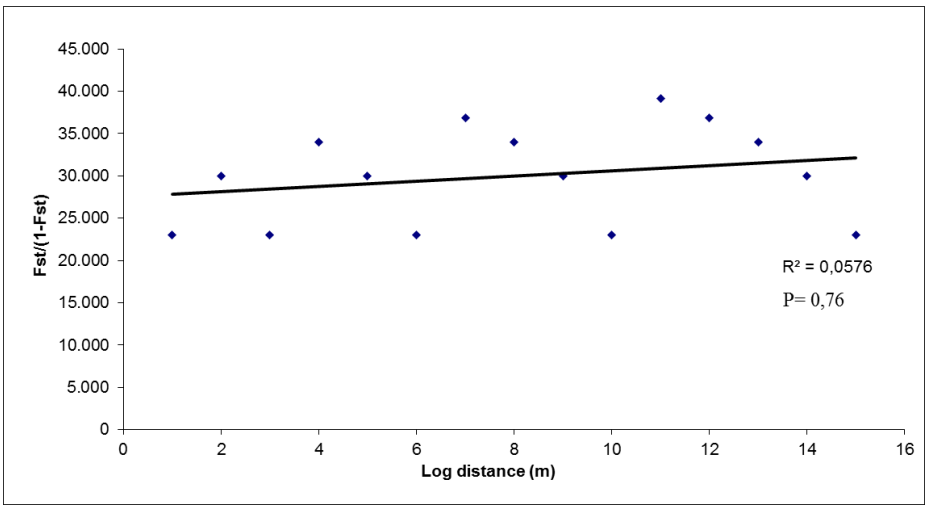

Figure 17. Relationship between genetic differentiation estimates and the logarithm of geographical distance between the *Eunicella singularis* populations.

The DAPC did not show major changes compared with the complete data set (Figure 18).

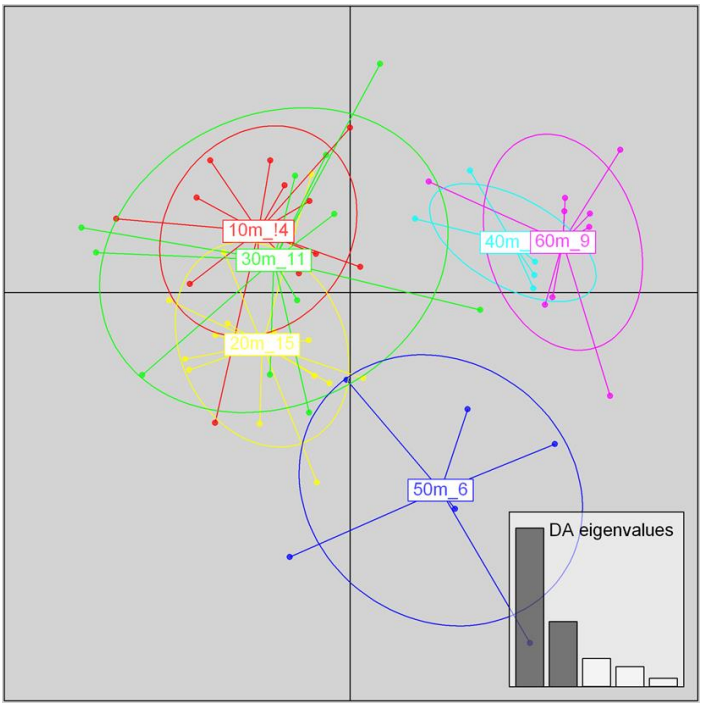

Figure 18. Plots of the first two axes obtained in the Discriminant Analysis of Principal Components using microsatellite dataset. Dots represent individuals.

5.2 Depth gradient according to ITS-1

The length of the amplified ITS-1 fragment was 200 pb, and across the 72 individuals sequenced showed 4 variable sites at the positions 21, 158, 159 and 173, defining 5 sequence types (Table 17). The more frequent sequence types was the ST1 and the ST4, which were present in 56 and 10 individuals, respectively (Table 18). Based on the electropherograms, one single double peak (unresolved peak) suggesting heterozygote individuals, was identified. In fact the PHASE analysis implemented in DNAsp showed that the individuals ES_40m_1, 6, 8 and 9 and individuals ES_60m_5 and ES_60m_8 were heterozygotes with two different sequence types: ST1 and ST4. Some of these heterozygotes presented different MLGs. ES_40m _1 presented the E MLG; ES_40m 8 and ES_40m_9 presented the C MLG; ES_60m_5 presented the A MLG. ES_20m and ES_60m populations presented three sequence types, while

ES_10m, ES_30m and ES_50m populations presented only one sequence type. ES_60m populations present the higher haplotype diversity (Table 18).

Sequence type	Variable site position 21	Variable site position 158	Variable site position 159	Variable site position 173
ST 1	T	T	T	A
ST 2	T	T	C	G
ST 3	T	T	C	A
ST 4	T	C	T	A
ST 5	C	T	T	A

Table 17. Sequences types and position of the variable sites.

	ES_10m	ES_20m	ES_30m	ES_40m	ES_50m	ES_60m
ST 1	12	9	11	6	12	5
ST 2	0	1	0	0	0	0
ST 3	0	1	0	0	0	0
ST 4	0	0	0	10	0	6
ST 5	0	0	0	0	0	2
N ST	1	3	1	2	1	3
Hd	0	0.3	0	0.46	0	0.65
Pi	0	0.0022	0	0.0023	0	0.0042

Table 18. Sequence types frequencies including heterozygotes individuals, haplotype diversity (in the sense of sequence type) and nucleotide diversity in each population. N ST: number of sequence types; Hd: haplotype diversity; Pi: nucleotide diversity.

The pairwise multilocus estimates of F_{ST} showed no significant differentiation between shallow (above 30 m depth) and deep populations (below 30m depth), neither between ES_50m population and shallow populations, or between ES_40m and ES_60 populations (Table 19).

	10 m	20 m	30 m	40 m	50 m	60 m
10 m	0.00					
20 m	0.10	0.00				
30 m	0.00	0.10	0.00			
40 m	**0.65**	**0.50**	**0.65**	0.00		
50 m	0.00	0.10	0.00	**0.65**	0.00	
60 m	**0.35**	**0.28**	**0.35**	0.07	**0.35**	0.00

Table 19. Pairwise multilocus estimates of F_{ST} (Weir & Cockerham 1984) between all the *Eunicella singularis* populations. Bold numbers are statistically significant at 0.01 after false discovery rate correction.

The AMOVA revealed that the 55.55% (P<0.001) of the total genetic variation occurred within populations. The percentage of the variation among populations within groups is also significant (12.08 %, P<0.01) (Table 20).

Source of variation	d.f	Sum of squares	Variance components	Significance tests	Percentage of variation
Among groups	1	5.149	0.0492	0.42913+-0.01210	19.35
Among populations within groups	4	6.609	0.06387	0.00000+-0.00000	25.10
Within populations	136	19.227	0.14138	0.00000+-0.00000	55.55
Total	141	30.986	0.25449		

Table 20. Analysis of molecular variance (AMOVA) based on two groups: above and below 30 meter depth.

The relationship between genetic differentiation and geographical distance based on the Mantel test was not statistical significant (P=0.59).

5.2.1 Preliminary phylogeny of the genus *Eunicella* based on ITS-1

An alignment of the ITS-1 fragment of 206 bp was obtained including 22 sequences of *Eunicella s. aphyta*, 5 sequences of *E. cavolinii*, 5 sequences of *E. verrucosa* and 6 sequences of *E. racemosa*. Among them 11 variable sites were identified at positions:

18-21, 102, 104, 109, 115, 118, 163, 173 and 178, that present 7 INDEL, that define 4 sequence types (Table 21). Based on electropherogram one single double peak (unresolved peak) was identified suggesting the existence of heterozygote individuals. The PHASE analysis implemented in DNAsp showed that the sample CAV 2 from the *E. Cavolinii* population presented 2 different sequence types: ST1 and ST_C. These low numbers of heterozygote individuals highlight the low variability inside and between taxa of the marker used. Furthermore all the populations presented one sequence type, except for *E. cavolinii*, which population presented 2 sequence types, due to the heterozygote individual. ST_V and ST_R were exclusive of *E. verrucosa* and *E. racemosa*, respectively (Table 22).

Sequence	Variable sites position																		
Types	18	19	20	21	-	-	-	-	-	102	104	109	115	118	163		178		
ST1	T	C	C	T	-	-	-	-	-	G	A	G	G	G	T	-	T	T	
ST_C	T	C	C	T	-	-	-	-	-	G	A	G	G	G	C	-	T	T	
ST_V	T	C	C	T	-	-	-	-	-	G	A	G	G	G	T	-	A	T	
ST_R	C	T	T	C	A	A	A	G	C	A	T	C	C	A	T	T	T	-	

Table 21. Sequence types and position of the variable sites.

	E.s. aphyta	***E. cavolinii***	***E. verrucosa***	***E. racemosa***
ST1	22	5	0	0
ST_C	0	2	0	0
ST_V	0	0	6	0
ST_R	0	0	0	6
N ST	1	2	1	1
Hd	0	0.409	0	0
Pi	0	0.002	0	0

Table 22. Number of sequence types, frequencies including heterozygotes individuals, haplotype diversity and nucleotide diversity of the 5 sequence types in each population. N ST: number of sequence types; Hd: haplotype diversity; Pi: nucleotide diversity.

The p-distance matrix showed that the greatest divergence among sequences was observed between *Eunicella racemosa* and all the other species. Moreover, the genetic distance observed within the populations of *Eunicella singularis* were comparable at those observed between *Eunicella singularis* and the other two *Eunicella* (*E. cavolinii* and *E. verrucosa*) (Table 23).

	ES_10m	ES_20m	ES_30m	ES_40m	ES_50m	ES_60m	*E.s. aphyta*	*E.cavolinii*	*E.verrucosa*	*E. racemosa*
ES_10m	0.00									
ES_20m	0.0012	0.00								
ES_30m	0.00	0.0012	0.00							
ES_40m	0.0025	0.0037	0.0025	0.00						
ES_50m	0.00	0.00125	0.00	0.0025	0.00					
ES_60m	0.0027	0.0039	0.0027	0.0023	0.0027	0.00				
E.s. aphyta	0.00	0.0012	0.00	0.0025	0.00	0.0027	0.00			
E.cavolinii	0.0008	0.0020	0.0008	0.0018	0.0008	0.0025	0.0008	0.00		
E.verrucosa	0.005	0.0062	0.005	0.0075	0.005	0.0077	0.005	0.0058	0.00	
E.racemosa	0.045	0.0464	0.0452	0.0478	0.0452	0.0461	0.0452	0.0461	0.0502	0.00

Table 23. Pairwise genetic *p*-distance among The *Eunicella* species (including the 6 populations of *Eunicella singularis*).

ITS-1 bayesian inference tree showed that the *Eunicella racemosa* ST (ST_R) was the most different (PP = 0.9635). Moreover, ST_ 1 clustered together with ST_V (with low Posterior probability) and ST_4 with ST_C (PP = 0.995) (Figure 19).

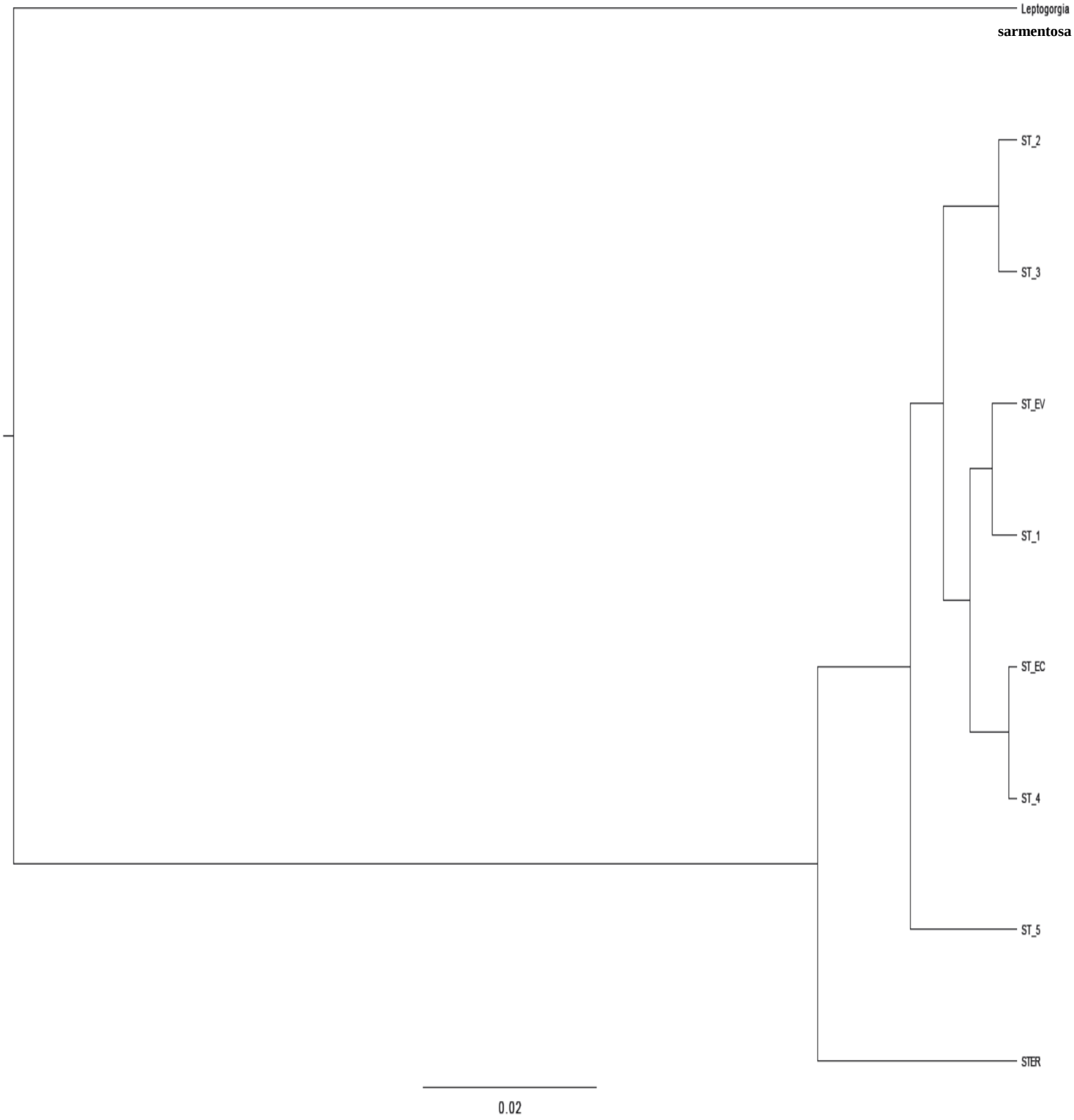

Figure 19. ITS-1 Bayesian Inference tree illustrating the phylogenetic relationships among the *Eunicella* species. Numbers near nodes are posterior probability (PP) values. ST1,2,3,4,5, are the sequence types observed in *E. singularis*; ST_C is the private ST of *E. cavolinii*; ST_V is the private ST of *E. verrucosa*; ST_R is the private ST of *E.racemosa*

5.3 Cross-species amplification of the microsatellite loci in the genus *Eunicella*

A total of 141 individuals were genotypes for the 5 loci: 99 individuals of *Eunicella singularis*, 26 individuals of *E. s.aphyta*, 5 individuals of *E. cavolinii*, 5 individuals of *E. verrucosa* and 6 individuals of *E. racemosa*. All the loci amplify for all the species. Moreover, all the species showed the same allelic range for each locus. Only, the locus C40 seems potentially useful to discriminate species since two private alleles (300 and 306) were observed in *E. verrucosa* (4 individuals), and one other private allele (282) were observed in *E. racemosa* (1 individual). The locus C21 showed no amplification for all the individuals from *E. stricta aphyta* and *E. racemosa* (Table 24).

	E. singularis	***E.s. aphyta***	***E. cavolinii***	***E. verrucosa***	***E. Racemosa***
	n (99)	n(26)	n(5)	n(5)	n(6)
c40	0	285	0	0	282
	285	291	285	285	285
	291	294	291	294	0
	294	0	0	0	0
	0	0	0	300	0
	0	0	0	306	0
s10	190	190	0	190	0
	196	196	196	196	196
	222	222	0	0	0
c30	187	0	0	0	0
	191	191	191	191	0
	199	199	0	199	199
c21	183	0	186	0	-
	189	0	0	189	-
	0	0	191	191	-
	195	195	0	195	-
	198	198	0	199	-
	201	0	0	0	-
caes64	179	0	179	179	0
	181	181	181	181	181

Table 24. Alleles per locus computed over all loci for each *Eunicella* species. n: number of individuals, - : null amplification.

6 DISCUSSION

This study investigated patterns of genetic variability and structuring of *Eunicella singularis* along a depth gradient from 10 m to 60 m depth. Moreover, genetic evidence of the two morphotypes and their systematic position within the genus *Eunicella* has been addressed by analyzing the phylogenetic relationships among *Eunicella* species. The main results were:

1) in *E. singularis* genetic variability decreases along a depth gradient;
2) significant genetic structuring was observed between shallow and deeper populations, with a threshold around 30 m depth;
3) phylogenetic pattern within the genus *Eunicella* are complex.

Estimates of multilocus polymorphisms found in *Eunicella singularis* using microsatellites were low. This result is confirmed also by the presence of several individuals sharing the same multilocus genotypes. Nevertheless, the presence of shared MLGs could be due to the low number of loci used, as suggested also by the high probability of identity, and/or to some bias occurred during the sampling (e.g. fragmentation of the colonies). This last hypothesis is not supported given that we found individuals sharing the same MLG in different populations. The levels of the genetic variability was low, the mean number of alleles per locus ranged between 1.6 and 3. Similar values has been reported in four *Eunicella singularis* populations at 2 different sites in France (Banyuls and Méjan) at two depth ranges (from -10 to -20 m and from -25 to -35m depth) (Abdoullaye et al. 2010). However, our results seem closer to those obtained by Pey et al. (2013) which reported a mean number of alleles per locus of 3-4 in two different sites (Medes island, Spain and Riou Island, France). One of their study site is very close to our study sites, and covers similar bathymetric range (10-40 m). Most populations, except those at 40 and 60 meters depth, showed a significant deviation of the HW equilibrium, emphasized by high positive F_{IS} estimates. This pattern might be due to processes that affect intrapopulational gene flow (e.g. inbreeding, Wahlund effect, and the presence and of a threshold between –40 and –50 m depth), but could also be due to technical problems, such as the presence of non-amplifying or null alleles. However, we can reject the null alleles origin, since

no null amplification was observed in the data set. This low polymorphism doesn't allow to clearly discriminate whether this pattern is due to biological and ecological features of the species, or to technical issues related to the loci used. The two populations in HW equilibrium are those that presented the higher number of individuals sharing the same MLG, nevertheless, using the reduced data set (excluding the csamples sharing the MLG) the results did not change. The analysis based on microsatellite loci showed a reduction of genetic variability of *E. singularis* along a depth gradient. A significant difference in terms of gene diversity and allelic richness was observed between populations above and below 30 meters depth. Similar reduction of genetic variability with depth has been already observed in other Mediterranean coral, *Corallium rubrum* (Costantini et al. 2011). Furthermore, also for this species a consistent reduction in genetic variability along a similar depth gradient was observed. Reduced genetic diversity is expected in small, isolated, and/or recently founded populations (Bradshaw et al. 2007). A reduction of gene diversity could also be due to differences in life-history, trophic ecology, biochemical pathways and physiology: Gori et al. (2012b) reported differences between shallow and deep populations of *Eunicella singularis* in terms of lipid content. Differences were attributed to seasonal variation in food availability, while lipid content is higher during the summer in shallow populations, it is lower and more constant in deep populations due both to the presence of symbiotic algae in shallow populations with an important role in lipid translocation and to a higher concentrations of phytoplankton and suspended material above the pycnocline. The present results show that populations above 30 m had a higher genetic variability than those living below 30 m. In fact, the pairwise comparisons between depths revealed that 30 m could act as a boundary between shallow- and deep-water populations. This differentiation does differ from the study by Pey et al. (2013), although they only considered populations up to 40 m depth. Our results, together with the significant genetic structuring observed at 30 m depth (see below), confirmed the presence of two genetically different *Eunicella singularis* morphotypes as suggested by Gori et al. (2012a). The presence/absence of symbiotic algae was the principal discriminating characteristic, but they also differed in color, colony shape and sclerite features. These two different morphological forms could be due to phenotypic plasticity, allowing populations to better fit in different

environmental conditions, or to a break in gene flow determining the isolation of the two populations and an accumulation of genetic differences. Pairwise F_{ST} estimates using microsatellite loci and ITS-1 sequence were not significantly different between populations above 30 m, while these populations were significantly different from those below 30 m. Conversely, pairwise F_{ST} between populations below 30 m were all significant. The reduced microsatellite dataset showed the same patterns, but in this case the -40m and -60m populations were not significantly different. The structure obtained with the ITS-1 showed no significant differences between the -50m population and the populations above -30 m. Both STRUCTURE analyses and Mantel tests suggested the absence of a vertical gradient of genetic structuring, due to the high genetic similarity between individuals from 40 m and 60 m depth. Abiotic factors vary with depth and determine different environmental conditions, so shallow waters are characterized by strong hydrodynamic forces, bidirectional currents (Riedl 1971) and high light irradiance, while deeper waters are affected by unidirectional water movement, lower light intensity, and only storm-induced waves which are usually very strong and can cause direct physical damage (Riedl 1971; Hiscock 1983). For *Eunicella singularis*, differences in water turbulence have been reported as the main cause of variability in colony shape (Théodor 1963; Skoufas et al. 2000). Moreover, in temperate seas, during the summer, high irradiance induces strong water column stratification that can cause a depletion of suspended material in shallower waters (Coma et al. 2000). In summer the thermocline ranges between 10 to 40 meters, and can affect larval dispersion, survival of the settlers/recruits, and food availability (Coma et al. 2000; Rossi and Gili 2005; Costantini et al. 2011). *E. singularis* reproduces in late May and June, when the water column is stratified (Ribes et al. 2007; Gori et al. 2007), so the recruitment of shallow populations can be restricted to substrates above the thermocline, whereas larvae from the deep populations remain below. Furthermore, shifts in the spawning period between shallow and deep populations could act as a barrier to gene flow. Recent studies reported a delay in the release of gametes with increasing depth (Weinberg and Weinberg 1979; Gori el al. 2007). Moreover, reproduction may be sensitive to fluctuations in food availability, lunar and solar phase, so the precise spawning time may be affected by the environmental conditions of the year (Tsounis et al. 2006b). In this study the analysis

of the genetic structure of these morphotypes using ITS-1 marker did not discriminated between shallow and deep populations of *E. singularis* because both populations showed the same variable sites. These results can be affected by the lower sample size of each population or by the very low variability of the marker used. To assess the phylogenetic relationship among the *Eunicella* genus a preliminary study was carried out using the sequence polymorphism of the ITS-1 region. The Bayesian Inference tree, showed how this marker was not able to discriminate the different Mediterranean *Eunicella* species. The sequences of *E. cavolinii* and *E. verrucosa*, clustered together with those of *Eunicella singularis*. Moreover, we found a similar genetic divergence within the *Eunicella singularis* populations compared to that observed among the three species. Again, the 10 individuals of *E. stricta aphyta* showed one single sequence type that corresponded to the more frequent sequence type occurred on *E. singularis* populations. The mitochondrial gene MSH1 is not helpful nor to discriminate between morphotypes of *E. singularis*, nor for the different species of *Eunicella* (Gori et al. 2012a). Similar results were obtained for the barcoding gene COI and the ITS2, which were identical in *E. singularis* and *E. cavolinii* (Calderon et al. 2006). This could mean that either all "species" of the genus *Eunicella* are in fact phenotypic variations of a single species, or that all of them have originated in a recent speciation events and in the genetic markers examined differences are not yet apparent. The ITS1 marker was only able to discriminate *Eunicella racemosa* from the others species. *E. racemosa* is the only Atlantic species analyzed, furthermore its shallow water habitat, around 15 m depth, and its colonies up to 2 meters high, make it the species with the more distinctive life habits and morphology. Similar results were obtained by Lopez-Gonzalez (unpublished) using the msh1, COI and Igr1. The msh1 of *E. racemosa* differs from the other *Eunicella* species in a single silent (the variants don't produce a change of aminoacid) substitution. The COI differs in four substitutions, one of which produces a change in an aminoacid in *E. racemosa* with respect to the other *Eunicella* species (Lopez- Gonzalez P, unpublished). The great morphological variability of *Eunicella* genus, as other Octocorallia genus, leads to considerable confusion regarding species boundaries and classification, challenging the methods and boundaries accepted for most species.

7 CONCLUSION

Eunicella singularis is a very important Mediterranean gorgonian since it has a role as engineer species providing biomass and complexity to coralligenous habitats. So, a better knowledge of the biotic and abiotic factors that can influence the growth, the stability and the connectivity of this species is required to understand how it will respond on different environmental conditions and to future natural and anthropogenic impacts. In this study the depth seems to influence the genetic variability of *E. singularis*. This pattern can be due to the great capability of morphological adaptation under different conditions or to the presence of a physical barrier, as the thermocline, that can lead to a decrease in gene flow and connectivity between shallow and deep populations. The microsatellites markers chosen to investigate the genetic variability were able to discriminate between shallow ad deep *Eunicella singularis* morphotypes. Replicated studies in other sites where *Eunicella singularis* is present in bathymetric range covered by this study will disentangle environmental factors (e.g. geography, geomorphology, temperature, salinity, etc.) from evolutionary processes of the species. Moreover, the microsatellite loci developed for *E. singularis* can be effectively used to study *Eunicella* species, highlighting their potential use in these vulnerable marine taxa. ITS sequences were not able to clarify completely the phylogenetic relationships among *Eunicella* species. *Eunicella racemosa* seems to be genetically more different compared to the Mediterranean species. Increasing the number of loci and the sample size, or finding of different more variable markers (e.g. 28S, Mac Fadden, Pers comm.), will be useful to better understand this pattern. Based on the results of this study we can conclude that 1) no molecular markers able to discriminate *Eunicella* species is available, ongoing speciation event occur and is not yet detected by the available genetic markers examined and/or 2) in the Mediterranean *Eunciella* includes only one species and within these species there is a large phenotypic variation. The accurate delineation of species and implementation of a proper taxonomy may profoundly improve our understanding of *Eunicella'*s reproductive biology, biogeographic distributions, and resilience to climate warming, information that must be considered when planning conservation and management of coralligenous habitat.

REFERENCES

- Abdoullaye, D. (2010). Permanent Genetic Resources added to Molecular Ecology Resources Database 1 August 2009–30 September 2009. Mol Ecol Res 10: 232–236

- Abel (1959). Zur Kenntniss der mariner Hohtenfauna unter besonderer Beruchsixhtigung der Anthozoen.- Pubbl. Staz. Zool. Napoli. 30 (suppl.): 1-94

- Agrawal, A.A. (2001). Phenotypic plasticity in the interactions and evolution of species. Science. 294: 321–32

- Aguilar, C., Sanchez, J.A. (2007). Phylogenetic hypotheses of gorgoniid octocorals according to ITS2 and their predicted RNA secondary structures. Mol Phylog. and Evol.. 3: 774-786.

- Balduzzi A., Bianchi C.N., Cattaneo-Vietti R et al. (1992). First data on the benthic communities around the Galli- naria Island (Ligurian Sea, NW Mediterranean). Proceedings of the 10th Meeting of the Italian Association of Oceanology and Limnology, Alassio. 603-617.

- Ballesteros, E. (2006). Mediterranean coralligenous assemblages: a synthesis of present knowledge. Oceanogr Mar Biol Annu Rev. 44:123–195

- Barbrook A.C., Visram S., Douglas A.E., Howe, C.J. (2006). Molecular diversity of dinoflagellate symbionts of cnidaria: the psbA minicircle of *Symbiodinium*. Protist 157:159–171. doi:10.1016/j

- Bavestrello, G., Cerrano, C., Zanzi, D., Cattaneo-Vietti, R., (1997). Damage by fishing activities to the Gorgonian coral *Paramuricea clavata* in the Ligurian Sea. Aq Con: Mar Fresh Ecosyst 7: 253–262.

- Best, M. B., Boekschoten, G. J. & Oosterbaan, A. (1984). Species concept and ecomorph variation in living and fossil scleractinia. Palae. AmE. 54: 70–79.

-Birkeland, C. (1974). The effect of wave action on the population dynamics of *Gorgonia ventalina* (Linnaeus). Stud Trop Oceanogr. 12:15–126

- Bo, M., Bavestrello, G., Canese, S., Giusti, M., Salvati, E., Angiolillo, M., Greco, S. (2009). Characteristics of a black coral meadow in the twilight zone of the central Mediterranean Sea. Mar Ecol Prog Ser. 397: 53–61

- Bo, M., Bertolino, M., Borghini, M., et al. (2011). Characteristics of the mesophotic megabenthic assemblages of the Vercelli seamount (North Tyrrhenian Sea). PLoS ONE 6:e16357

- Bongaerts, P., Ridgway, T., Sampayo, E.M., Hoegh-Guldberg, O. (2010). Assessing the 'deep reef refugia' hypothesis: focus on Caribbean reefs. Coral Reefs. 29: 309–327

- Bradshaw, A. D. (1965). Evolutionary significance of phenotypic plasticity in plants.Adv. in Gen. 13: 115–155

- Bramanti, L., Iannelli, M., Santangelo, G. (2009). Mathematical modeling for conservation and management of gorgonian corals: youngs and olds, could they coexist? Ecol Model. 220:2851–2856

- Brazeau, D. A., D. F. Gleason, and M. E. Morgan. (1998). Self fertilization in brooding hermaphroditic Caribbean corals: Evidence from molecular markers. J. Exp. Mar. Biol. Ecol. 231: 225–238.

- Calderon, I., Garrabou, J., Aurelle, D. (2006). Evaluation of the utility of COI and ITS markers as tools for population genetic studies of temperate gorgonians. J Exp Mar Biol Ecol. 336: 184–197.

- Carlon, D.B., Budd, A.F. (2002). Incipient speciation among a depth gradient in a scleractinian coral?. Evolution. 56: 2227-2242

- Carpine, C., Grasshoff, M. (1975). Les gorgonaires de la Mediterranee. Bull Inst Oceanogr Monaco. 71:1–140

- Cocito S., Bedulli D., Sgorbini S. (2002). Distribution patterns of the sublittoral epibenthic assemblages on a rocky shoal in the Ligurian Sea (NW Mediterranean). Sci. Mar. 66(2): 175-181

- Coma, R., Linares, C., Ribes, M., Diaz, D., Garrabou, J., Ballesteros, E. (2006), Consequences of a mass mortality in populations of Eunicella singularis (Cnidaria: Octocorallia) in Menorca (NW Mediterranean). Mar Ecol Prog Ser. 327: 51–60

- Coma, R., Pola, E., Ribes, M., Zabala, M., (2004). Long-term assessment of temperate octocoral mortality patterns, protected vs. unprotected areas. Ecol Appl. 14: 1466–1478.

- Coma, R., Ribes, M., Zabala, M., Gili, J.M. (1998). Growth in a modular marine invertebrate. Estuar Coastal Shelf Sci. 47: 459-47

- Costantini, F., Carlesi, L., Abbiati, M. (2013). Quantifying spatial genetic structuring in mesophotic populations of the Precious Coral *Corallium rubrum*. PloS one. 8: e61546.

- Dayton, P. K., S. F. Thrush, M. T. Agardy, and R. J. Hofman (1995). Environmental effects of marine fishing. Mar. and Freshwater Cons. 5:205–232.

- DeGeest, A.L., Mullenbach, B.L., Puig, P., Nittrouer, C.A., Drexler, T.M., Durrieu de Madron, X., Orage, D.L. (2008). Sediment accumulation in the western Gulf of Lions, France: the role of Cap de Creus canyon in linking shelf and slope sediment dispersal systems. Cont Shelf Res. 28: 2031–2047

- Foster, A. B. (1979). Phenotypic plasticity in the reef corals *Montastrea annularis* and *Siderastrea siderea*. J Exp Mar Biol Ecol. 39: 25–54.

- France, S.C., Khocher,T.D. (1996). Geographic and bathimetric gradient of mithocondrial 16S rRNA sequence divergence among deep.sea amphipods, *Euritenes gryllus*. 12: 633-643

- Garrabou, J., Coma, R., Bensoussan, N.,et al. (2009). Mass mortality in Northwestern Mediterranean rocky benthic communities: effects of the 2003 heat wave. Global Change Biol .15:1090–1103

- Garrabou, J., Harmelin, J.G. (2002). A 20-year study on life-history traits of a harvested long- lived temperate coral in the NW Mediterranean: insights into conservation and management needs. J Anim Ecol. 71:966–978

- Garrabou, J., Harmelin, J.G. (2002). A 20-year study on life-history traits of a harvested long-lived temperature coral in the NW Mediterranean: insight into conservation and management needs. J. Anim. Ecol. 71: 966-978

- Gili, J.M., Coma, R. (1998). Benthic suspension feeders: their paramount role in littoral marine food webs. Tree. 13:316–321

- Gori, A., Bramanti, L. (2012a). Characterization of the zooxanthellate and azooxanthellate morphotypes of the Mediterranean gorgonian *Eunicella singularis*, Mar Biol. 159: 1485-1496

- Gori, A., Linares, C., Rossi, S., Coma, R., Gili, J.M. (2007). Spatial variability in reproductive cycle of the gorgonians *Paramuricea clavata* and *Eunicella singularis* (Anthozoa, Octocorallia) in the Western Mediterranean Sea. Mar Biol. 151:1571–1584

- Gori, A., Rossi, S., Berganzo, E., Pretus, J.L., Dale, M.R.T., Gili, J.M. (2011a). Spatial distribution patterns of the gorgonians *Eunicella singularis, Paramuricea clavata* and *Leptogorgia sarmentosa* (Cape of Creus, Northwestern Mediterranean Sea). Mar Biol. 158:143–158

- Gori, A., Rossi, S., Linares, C., Berganzo, E., Orejas, C., Dale, M.R.T., Gili, J.M. (2011b). Size and spatial structure in deep versus shallow populations of the Mediterranean gorgonian *Eunicella singularis* (Cap de Creus, northwestern Mediterranean Sea). Mar Biol. 158:1721–1732

- Gori, N. Viladrich (2012b). Reproductive cycle and trophic ecologyin deep versus shallow populations ofthe Mediterranean gorgonian *Eunicella singularis* (Cap de Creus, northwesternMediterranean Sea). Coral Reefs. 31.

- Grajales,A., Aguilar, C., Sánchez, J.A. (2007). Phylogenetic reconstruction using secondary structures of Internal Transcribed Spacer 2 (ITS2, rDNA): finding the molecular and morphological gap in Caribbean gorgonian corals. BMC Evol Biol. 2007, 7:90

- Grasshoff, M. (1992). Die Flachwasser-Gorgonarien von Europa und Westafrika (Cnidaria. Anthozoa). Courier Forschunginsiiiui Senckenberg. 149:1–135

- Grigg, R.W. (1977). Population dynamics of two gorgonian corals. Ecology. 58:278–290

- Harmelin, J.G., Bachet, F., and Gracia, F. (1995). Mediterranean marine reserves: fish indices as tests of protection efficiency. Mar Ecol. 16:233–250.

- Harmelin, J.G., Marinopoulos, J. (1994). Population structure and partial mortality of the gorgonian *Paramuricea clavata* (Risso) in the north-western Mediterranean (France, Port-Cros Island). Marine Life. 4: 5–13

- Hellberg, M.E. (2006). No variation and low synonymous substitution rates in coral mtDNA despite high nuclear variation. BMC Evol Biol. 6: 24.

- Hendry, A.P., Taylor ,E.B. (2004). How much of the variation in adaptative divergence can be explained by gene flow? An evaluation using lake-stream stickleback pairs. Evolution. 10: 2319–2331

- Hiscock, K. (1983). Water movement. In: Earll E, Erwin DG (eds) Sublittoral ecology. The ecology of the shallow sublittoral benthos. Oxford University Press, Oxford. 58–96

- Hoeksema, B. W. (1993). Phenotypic corallum variability in recent mobile reef corals. Courier Forschungsinstitut. 164: 263–272.

- Jaubert, J. (1977). Light, metabolism and growth forms of the hermatypic scleractinian coral *Synaraea convexa* in the lagoon of Moorea (French Polynesia). Proceedings of the Third International Coral Reef Symposium. 1: 483–488.

- Kawecki, T.J., Ebert ,D. (2004). Conceptual issues in local adaptation. Ecol. Lett. 7:1225–41

- La Greca, M. (1992), Zoologia degli invertebrati. La Greca.

- Lasker, H.R. (1991). Population growth of a gorgonian coral: equilibrium and non-equilibrium sensitivity to changes in life history variables. Oecologia. 86:503–509.

- Le Goff-Vitry, Pybus, O.C., Rogers A.D. (2004). Genetic structure of the deep-sea coral *Lophelia pertusa* in the northeast Atlantic revealed by microsatellites and internal transcribed spacer sequences. Mol Ecol. 3: 537-549.

- Linares, C., Coma, R., Garrabou, J., Dı´az, D., Zabala, M. (2008). Size distribution, density and disturbance in two Mediterranean gorgonians: *Paramuricea clavata* and *Eunicella singularis*. J Appl Ecol. 45:688–699

- Linares, C., Doak, D.F. (2010). Forcasting the combined effects of disparate disturbances on the persistence of long-lived gorgonians: a case study of *Paramuricea clavata*. Mar Ecol Prog Ser. 402: 59-68

- Linares, C., Doak, D.F., Coma, R., Dı´az, D., Zabala, M. (2007). Life history and viability of a long-lived marine invertebrate: the octocoral *Paramuricea clavata*. Ecology. 88:918–928

- Linares,C., Zabala, M. (2008). Size distribution, density and disturbance in two Mediterranean gorgonians: *Paramuricea clavata* and *Eunicella singularis*. J Appl Ecol. 45: 688-699.

- Merilä, J., Crnokrak, P. (2001). Comparison of genetic differentiation at marker loci and quantitative traits. J Evol Biol. 6: 892–903

- Meroz, E., Brinckner, I., Loya, Y., Retzman-Shemer, A. & Ilan, M. (2002). The effect of gravity on coral morphology. Proc. R. Soc. B. 269: 717–720

- Miller, K. J. (1994). Morphological variation in the coral genus Platygyra: environmental influences and taxonomic implications. Mar Ecol Prog Series. 110: 19–28.

- Millot, C. (1990). The Gulf of Lions' hydrodynamics. Cont Shelf Res. 19:885–894

- Mistri, M., Ceccherelli, V. (1993). Growth of the Mediterranean gorgonian *Lophogorgia ceratophyta* (L., 1758). PSZN I. Mar Ecol. 14: 329–340

- Muko, S., Kawasaki, K., Sakai, K., Takasu, F. & Shigesada, N. (2000). Morphological plasticity in the coral *Porites sillimaniani* and its adaptive significance. Bul Mar Sci. 66: 225–239.

- Newman, R. A. (1992). Adaptive plasticity in amphibian metamorphosis. Bioscience. 42: 671–678.

- Pax, F. and I. Muller (1962). Mechanical properties of the axial skeleton in gorgonians. Coral Reefs. 5: 213–219

- Pey, A. (2013). Thermal threshold and sensitivity of the only symbiotic Mediterranean gorgonian *Eunicella singularis* by morphometric and genotypic analyses. C. R. Biologies. 7: 311-341

- Poliseno, A. (2007). Filogenesi molecolare di Ottocoralli Mediterranei, Tesi di Laurea, Università di Bologna

- Prada, C. (2008). Phenotypic plasticity or speciation? A case from a clonal marine organism. BMC Evol Biol. 8:47.

- Rex, M. A., Etter, R. J., Stuart, C. T. (1997). Large-scale patterns of species diversity in the deep-sea benthos. Marine Biodiversity: Patterns and Processes. Cambridge University Press, Cambridge. 94-121.

- Ribes, M., Coma, R., Rossi, S., Micheli, M. (2007). Cycle of gonadal development in *Eunicella singularis* (Cnidaria: Octocorallia): trends in sexual reproduction in gorgonians. Invertebr Biol. 126:307–317

- Riedl (1966). Biologie der Meereshohlen. I- 636 - Rossi, L. (1959). Le specie di *Eunicella* (Gorgonaria) del Golfo di Genova. Res Ligusticae. 118:203–225.

- Rodriguez-Lanetty, M., Hoegh-Guldberg, O. (2002). The phylogeography and connectivity of the latitudinally widespread scleractinian coral *Plesiastrea versipora* in the Western Pacific. Mol Ecol. 7: 1177-1189.

- Rossi, S., Gili, J.M. (2009). The cycle of gonadal development of the soft bottom-gravel gorgonian *Leptogorgia sarmentosa* in the NW Mediterranean Sea. Invertebr Reprod Dev. 53:175–190

- Rossi, S., Tsounis, G., Orejas, C., Padro´n, T., Gili, J.M., Bramanti, L., Teixido´, N., Gutt, J. (2008). Survey of deep-dwelling red coral (*Corallium rubrum*) populations at Cap de Creus (NW Mediterranean). Mar Biol. 154: 533–545

- Sánchez, J. A. (2007). Phenotypic plasticity and morphological integration in a marine modular invertebrate, BMC Evol Biol. 7: 122.

- Sanford, E., Kelly, M.W. (2010). Local adaptation in marine invertebrates. Annu. Rev. Mar. Sci. 2011. 3: 509–35

- Santangelo G, Bramanti L, Iannelli M (2007). Population dynamics and conservation biology of the over-exploited Mediterranean red coral. J Theor Biol. 244:416–423

- Scheiner , S. M. (1993). Genetics and the evolution of phenotypic plasticity. Annu. Rev. Ecol. Syst. 24: 35–68.

- Schlichting, C. D.; Pigliucci, M. (1998). Phenotypic evolution: a reaction norm perspective. 12

- Schlinchting, C. D. (1986). The evolution of phenotypic plasticity in plants. Annu. Rev. Ecol. Syst. 17: 667–693

- Schmalhausen, I.I. (1986). Factors of evolution: the theory of stabilizing selection. University of Chicago Press, Chicago.

- Schmidt-Roach, S. (2013). Assessing hidden species diversity in the coral *Pocillopora damicornis* from Eastern Australia, Coral Reefs. 32: 161-172.

- Shaish, L., Abelson, A. & Rinkevick, B. (2007). How plastic can phenotypic plasticity be? The branching coral - *Stylophora pistillata* as a model system. Public Library of Science ONE. 2: e644.

- Shashar, N., Kinane, S., Jokiel, P. L. and Patterson, M. R. (1996). Hydro mechanical boundary layers over a coral reef. J Exp Mar Biol Ecol. 199: 17–28.

- Shearer, T.L., Coffroth, M.A. (2006). Genetic identification of Caribbean scleractinian coral recruits at the Flower Garden Banks and the Florida Keys. Mar Ecol Prog Ser. 306: 133–142

- Shearer, T.L., Van Oppen ,M.J.H., Romano, S.L., Worheide, G. (2002). Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). Mol Ecol. 11: 2475–2487.

- Skoufas, G. (2006). Comparative biometry of *Eunicella singularis* (Gorgonian) sclerites at East Mediterranean Sea (North Aegean Sea, Greece). Mar Biol. 149:1365–1370

- Slatkin, M. (1995). A measure of population subdivision based on microsatellite allelic frequencies. Department of Integrative Biology, University of California, Berkeley, California 94720.

- Stearns , S. C. (1989). The evolutionary significance of phenotypic plasticity. Bioscience. 39: 436–446

- Théodor (1971) Phenomenes d'histo-incompatibilitè allogenique chez des invertebres diploblastique. Reactions letales entre organimes unicellulaires. I-VI, 1-176

- Théodor J (1963). Contribution a l'etude des gorgones (III): Trois formes adaptatives d' *Eunicella stricta* en fonction de la turbulence et du courant. Vie Milieu. 14: 815–818

- Théodor, J. (1969). Contribution a l'etude des gorgones (VIII): *Eunicella stricta aphyta* sous-espe`ce nouvelle sans zooxanthelles proche d'une espe`ce normalement infeste´e par ces algues.

- Todd, P. (2008). Morphological plasticity in scleractinian corals, Biological rewiew. 83: 315-317.

- Ulses, C., Estournel, C., Bonnin, J., Durrieu de Madron, X., Marsaleix, P. (2008). Impact of storms and dense water cascading on shelfslope exchanges in the Gulf of Lion (NW Mediterranean).

- Velimirov, B. (1973) Orientation in the sea fan *Eunicella cavolinii* related to water movement.- Helgol.Wiss. Meeresunters. 24: 163-173

- Veron, J. E. N. (1986). Corals of Australia and the Indo-Pacific. Angus and Robertson Publishers, Sydney.

- Veron, J. E. N. (2000). Corals of the world. Australian Institute of Marine Science, Townsville, Australia.

- Virgilio M, Airoldi L, Abbiati M (2006). Spatial and temporal variations of assemblages in a Mediterranean coralligenous reef and relationships with surface orientation. Coral Reefs. 25: 265-272.

- von Koch (1887). Die Gorgoniden des Golfes von Neapel und der angrenzenden Meeresabschinitte. Erster Theil einer Monographie der Anthozoa Alcyonaria. Fauna Flora Golf. Neapel. 15: 1-99

- Weinbauer, M., Velimirov, B. (1996b). Population dynamics and overgrowth of the sea fan *Eunicella cavolinii* (Coelenterata: Octocorallia). Estuar Cstl Shelf Sci. 42:583–595

- Weinberg, S. (1976) Revision of the common Octocorallia of the Mediterranean circalittoral. I. Gorgonacea. Institute of Taxonomic Zoology.

- Weinberg, S., Weinberg, F. (1979). The life cycle of a gorgonian: *Eunicella singularis* (Esper, 1794). Bijdr Dierkd. 48: 127–140

- Yoshioka, P.M., Yoshioka, B.B. (1991). A comparison of the survivorship and growth of shallow-water gorgonian species of Puerto Rico. Mar Ecol Prog. 69: 253–260.