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Ph.D. IN BIOLOGIA

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Genetic variability of macroalgae of the genus *Cystoseira* in the Gulf of Naples and analysis of the associated molluscs community

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ABSTRACT

The brown macroalgae of the genus *Cystoseira* are amongst the most important ecosystem engineering species along rocky coasts of the Mediterranean Sea establishing structurally complex and diversified habitat. Over the last few decades the disappearance of *Cystoseira* species has been recorded in wide geographical area as a consequence of anthropogenic impacts.

In the Gulf of Naples a recent study to outline historical changes in macroalgal diversity highlighted a drastic decrease of *Cystoseira* species in the intertidal zones. The decline seems to be largely related to the habitat destruction.

In order to assess the consequences of the current process of *Cystoseira* population fragmentation in the Gulf of Naples at species, population and community level and to provide tools for restoration and coastal management strategies, a multi-approach has been used. The diversity of the genus *Cystoseira* along the coasts of the Gulf of Naples has been investigated at species, genetic and ecosystem level.

The species have been genetically characterized through the analysis of the plastidial psbA gene. Eight microsatellites and the RADSeq, a next-generation sequencing method, have been employed to test their usefulness for connectivity and population genetic studies.

Overall *Cystoseira* associations in the Gulf of Naples show different pattern of genetic variability among and within the species. *Cystoseira amentacea* and *Cystoseira crinita* are more variable in terms of polymorphic sites and number of haplotypes compared to *Cystoseira compressa* and this seems to be related to the evolutionary history of these species rather than to their resilience to the environmental conditions.

The molluscs community associated with three *Cystoseira* species have been characterized and the different pattern of associated diversity have been evaluated.

The analysis at community level highlighted the importance of *Cystoseira* species as nursery for the recruitment of molluscs since only juvenile stages were found. Although the dominance of the bivalve *Mytilus galloprovincialis*, it is possible to identify some differences in the pattern of association of molluscs community. The three *Cystoseira* stands harbor a species-rich malacofauna assemblage, a total of 53 mollusc species were identified.

The present study outlines the importance of using a multi-approach in the analysis of diversity at different scales of investigation.

Moreover the results from the present study might be taken as an incentive for a series of protection and management strategies towards these important habitat forming species.

ABSTRACT

Le macroalghe brune del genere *Cystoseira* sono considerate tra le più importanti 'specie ingegnere' lungo le coste rocciose del Mar Mediterraneo dove costituiscono habitat complessi e diversificati. Negli ultimi decenni, diversi studi hanno registrato la scomparsa delle specie del genere *Cystoseira* in ampie zone geografiche a causa degli impatti di natura antropica.

Un recente studio sui cambiamenti storici nella diversità macroalgale nel Golfo di Napoli, ha evidenziato una drastica perdita di specie del genere *Cystoseira* soprattutto nella zona intertidale. Tale declino sembra essere legato alla distruzione dell'habitat naturale.

Al fine di stabilire le conseguenze dell'attuale processo di frammentazione delle popolazioni a *Cystoseira* nel Golfo di Napoli a livello di specie, popolazione e comunità, nel presente lavoro di tesi è stato utilizzato un multi-approccio. La diversità del genere *Cystoseira* lungo le coste del Golfo di Napoli è stata analizzata a livello specifico, genetico e più in generale a livello di ecosistema.

Le specie algali sono state caratterizzate da un punto di vista genetico mediante l'utilizzo del gene plastidiale psbA. Otto microsatelliti e la RADSeq, un approccio di sequenziamento di nuova generazione, sono stati testati al fine di comprenderne l'utilità negli studi di connettività e di genetica di popolazione.

In generale i popolamenti algali a *Cystoseira* nel Golfo di Napoli mostrano un diverso livello di variabilità genetica intra ed inter-specifico. Le specie *Cystoseira amentacea* e *Cystoseira crinita* sono più variabili in termini di siti polimorfici e numero di aplotipi rispetto alla specie *Cystoseira compressa*. Tale diversità sembra essere legata alla storia evolutiva delle suddette specie piuttosto che alla loro resilienza nei confronti delle condizioni ambientali.

La comunità di molluschi associata a tre specie del genere *Cystoseira* è stata caratterizzata ed è stata valutata la relativa diversità di composizione e struttura.

L'analisi a livello di comunità ha evidenziato l'importanza delle specie *Cystoseira* come nursery per il reclutamento di stadi giovanili di molluschi. Nonostante la dominanza del bivalve *Mytilus galloprovincialis*, la comunità di molluschi associata alle tre diverse specie algali è ben strutturata e diversificata. Le tre specie di *Cystoseira* ospitano una malacofauna molto ricca in termini di numero di specie (53 specie associate).

Il presente studio mette in luce l'importanza di un approccio integrato nell'analisi della diversità con vari livelli di indagine.

Inoltre tali risultati sono da considerarsi come incentivo per una serie di strategie di protezione e di gestione di queste importanti specie strutturanti l'habitat.

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CHAPTER I

GENERAL INTRODUCTION

I.1 Biological diversity or biodiversity

I.1.1 Definition

The word 'biodiversity' is a modern contraction of the term biological diversity that means the range of variation or variety within and among the living organisms.

The term was widely adopted from the 1980s when it came into common usage in science and environmental policy although its definition have been much elaborated and debated in the last three decades and its deep meaning is often misunderstood. Thomas Lovejoy is considered to be the 'Godfather of Biodiversity' since he introduced for the first time the term 'biological diversity' to the scientific community in 1980. However the original extended version provided by Lovejoy was possibly the simplest definition for biodiversity, lacking in specificity or context, it is merely defined as the number of species. Many authors disagreed with this definition since the term for this measure is instead the species richness (Fiedler and Jain 1992). The combined term 'biodiversity' has evidently been coined by Walter G. Rosen in 1985 for the first planning conference of the 'National Forum on Biological Diversity' organized by the National Research Council (NRC) in Washington. Edward O. Wilson launched the word into general use in 1986 in the proceedings of that forum (Harper and Hawksworth 1994).

The Convention on Biological Diversity which was signed within the United Nations Conference on Environment and Development (UNCED) at Rio de Janeiro in 1992 defined the internationally accepted definition of biological diversity as "*the variability among living organisms from all sources, including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part: this includes diversity within species, between species and of ecosystems*".

DeLong (1996) proposed a more comprehensive and detailed explanation, he defined the biodiversity as "*an attribute of an area and specifically refers to the variety within and among living organisms, assemblages of living organisms, biotic communities, and biotic processes, whether naturally occurring or modified by humans*". Moreover DeLong stated that "*biodiversity can be measured in terms of genetic diversity and the identity and number of different types of species, assemblages of species, biotic communities, and biotic processes, and the amount (e.g., abundance, biomass, cover, rate) and structure of each. It can be observed and measured at any spatial scale ranging from microsites and habitat patches to the entire biosphere*". An advantage of this definition is that it allows for modification according to the context in which it is used and presents a unified view of the traditional types of biological variety previously identified.

Various authors have proposed specific and detailed elaborations of this definition. Gaston and Spicer (1998) proposed a three-fold definition of 'biodiversity' including organismal or species diversity, genetic diversity and ecological or ecosystem diversity. Within each, the elements are organized in nested hierarchies, with those higher order elements comprising lower order (Gaston 2010) (Table I-1).

However, biodiversity does not have a universally agreed definition and is often redefined by the authors according to the context and purpose.

Table I-1. Elements of biodiversity (focusing on those levels that are most commonly used). Source: Gaston (2010)

Organismal diversity	**Ecological diversity**	**Genetic diversity**
Domains or Kingdoms	Biogeographic realm	
Phyla	Biomes	
Families	Provinces	
Genera	Ecoregions	
Species	Ecosystems	
Subspecies	Habitats	
Populations	Populations	Populations
Individuals		Individuals
		Chromosomes
		Genes
		Nucleotides

I.1.2 Species or organismal diversity

Historically the species are the fundamental descriptive units of the living world (Heywood and Baste 1995). Organismal diversity encompasses the full taxonomic hierarchy and its components, from kingdoms to individuals.

This kind of diversity is based on morphological and physiological features. The contribution of molecular techniques are constantly improving the species discrimination, for this reason the taxonomy is a discipline in continuous improvement.

Measures of organismal diversity are often based on the number of living species or species richness and their relative abundances (Pielou 1977). However the number of individuals on earth remains still difficult to assess first because the same species could be known under

more than one name (synonymy) and second because one name might encompass multiple species especially when those are closely related and look very similar (cryptic species) (Gaston 2010).

I.1.3 Ecological diversity

Ecological diversity is described by Gaston (2010) as that groups together all ecological scales from the population to biomes and biogeographic realm, including habitat and ecosystem. The assessment of this diversity is arguable since the boundaries between the different ecological elements are difficult to distinguish and conceptualize and also because some of the elements of ecological diversity clearly have both abiotic and biotic components. However, this ecological diversity makes it possible to evaluate the importance of habitat and the ecosystem in preserving biodiversity.

I.1.4 Genetic diversity

Genetic diversity includes all the components that characterize the genetic make-up of an organism (nucleotides, genes, chromosomes) (Hughes et al. 2008).
It is defined as the total number of genetic variation between individuals within a population and between populations.
Within a species, genetic diversity is commonly measured as follows:

- allelic richness: the average number of alleles per locus
- allelic diversity: the variety of alleles and their frequency
- genotypic richness: the number of genotypes within a population, it can be measured as the number of haplotypes
- gene diversity: the proportion of polymorphic loci across the genome
- heterozygosity: the average proportion of loci that carry two different alleles at a single locus within an individual
- nucleotide diversity (π): the average number of nucleotide differences per site between two random individuals selected from a population.

Genetic diversity is generated by mutation or introduced by migration (Frankham et al. 2002). It is commonly assessed through molecular markers such as microsatellites, AFLPs, direct mitochondrial, plastidial or nuclear DNA sequencing, and single-nucleotide polymorphisms (SNPs).
Genetic diversity plays an important role in the survival and adaptability of a species to environmental condition (Frankham 2005).

I.2 Biodiversity status

The incomplete sampling of the world's biodiversity together with a lack of strong extrapolation approaches make it difficult to estimate how many species there are on Earth. Mora et al. (2011) estimated about 8.7 million species of which about 2.2 million are marine, although about 86% of the species on Earth, and 91% in the ocean, still await description. The taxonomic experts suggested the range of 3 to 100 million species, however these predictions only focus on specific groups (May 2010).

The current comprehensive data-based catalogue of all known species of organism on Earth accounts for 1.635.200 living and 5.719 extinct species (Mora et al. 2011, Roskov et al. 2016).

I.3 Biodiversity loss

Terrestrial and marine biodiversity is decreasing at unprecedented rate as a result of the influence of human activities (Baillie et al. 2004). The International Union for Conservation of Nature (IUCN) estimates that nowadays 70% of known plant species, 22% of mammals, 32% of all amphibians and 12% of birds are threatened with extinction (IUCN; iucnredlist.org, last accessed January 2017).

In 500 million years, Earth has faced five major mass extinctions that have led to large and sudden drops in biodiversity (Wake and Vredenburg 2008). Extinction caused directly or indirectly by humans are occurring at a worrisome rate that far exceeds the natural process of extinction and may suggest that we are currently experiencing a sixth mass extinction (Leakey and Lewin 1996). What distinguishes the current extinctions from the previous ones is the responsibility of man in the disappearance of species.

Most of the documented extinctions have been of terrestrial species, followed by freshwater and marine. Recently there has been increasing attention in the scientific community that a broad range of marine species could be threatened of extinction and that marine biodiversity is experiencing potentially irreversible loss. Although governmental and public interest in marine conservation planning and policy is increasing, the information needed are seriously lacking (Polidoro et al. 2008).

The entity of threats in the marine systems are poorly understood mostly because marine species have long been considered resilient to extinction thus they have not been taken into account within extinction risk assessments (Webb and Mindel 2015). Habitat destruction and associated degradation and fragmentation, introduced and invasive alien species, overexploitation or indirectly climate change play a key role in the loss of marine

biodiversity (Worm et al. 2006). Often synergistic, these threats have substantially degraded marine biodiversity, with greater impacts predicted for the future (Sala and Knowlton 2006). Dulvy et al. (2003) documented 133 local and global extinctions of marine species including seabirds, marine mammals, fishes, invertebrates and seaweeds.

In 2006, IUCN, Conservation International and Old Dominion University initiated an ambitious project, the Global Marine Species Assessment to complete IUCN Red List assessments for a large number of marine species. Although only a fraction of marine taxa have been assessed, 1.206 marine species are currently classified as Critically Endangered, Endangered or Vulnerable on the IUCN Red List. These include 39% of assessed marine mammals, 33% of reef building corals, 20% of assessed marine birds, 19% of assessed mangroves and 17% of assessed seagrasses (Webb and Mindel 2015).

However, the conservation status of the majority of marine species has not yet been investigated on a global scale, an example is given by seaweeds. Most people, including many phycologists, do not immediately think of algae when discussing endangered or recently extinct species.

The first documented case of a historical extinction of an alga, *Vanvoorstia bennettiana* (Harvey) Papenfuss (Delesseriaceae, Rhodophyta), was reported by Millar (2003). Seaweeds have not been the focus for any Red List activity, however there are probably many species in these groups that are facing extinctions (Baillie et al. 2004). There are fewer than 400 other marine species that have been assessed for The IUCN Red List, of these, approximately 200 are marine fishes, 100 are marine molluscs and 75 are seaweeds. (Polidoro et al. 2008) (Figure I-1).

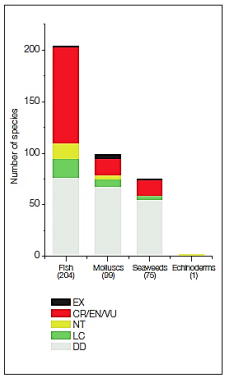


Figure I-1. Summary of 2008 Red List Categories for uncompleted clades of marine species. Number of species assessed in each group in parentheses. Source Polidoro et al. (2008)

I.4 The choice of the species to be preserved

With an ever-increasing number of species at risk, priorities and choices are needed to monitor and manage every aspect of biodiversity. For a long time the choice of species selected for conservation policy has focused on species that are emblematic for the ecosystems.

For example the 'flagship species' defined by Simberloff (1998) et Caro et al. (2004) as charismatic species, used as the focus of a broader conservation marketing campaign because they encourage public interest and sympathy (i.e. Bengal tiger *Panthera tigris*, giant panda *Ailuropoda melanoleuca*, Asian elephant *Elephas maximus*).

The 'umbrella species' (Simberloff 1998, Roberge and Angelstam 2004), species that needs such demanding habitat and large area requirements that saving them will automatically save many other species (i.e. old growth forest, Siberian tiger *Panthera tigris altaica*).

The great difficulty of species conservation management is to find the correct balance that allows the preservation of the species without damaging the others. During the years, the conservation policy has shifted from the protection of single species to the perspective to protect the whole ecosystem. The aim is to retain the ecological role of species within the ecosystem. The protection of the ecosystem as a whole seems to be the best solution of species conservation management (Simberloff 1998, Carignan and Villard 2002). The priority of conservation could be linked to that species having a central role in the stability of ecosystems such as keystone species, *sensu* Paine (1969) and engineering species, *sensu* Jones et al. (1994). These concepts are not fixed and are constantly changing.

I.4.1 The keystone species

In 1969, Paine defined the 'keystone species' as that "*species whose removal, whether natural or not, brings significant changes in population density and leading to a profound change in the ecosystem*". Given that there are many historical definitions of the keystone species, a list of examples best illustrates this concept. As described by Paine (1966), the sea stars *Pisaster ochraceus* may prey on sea urchins, mussels and other shellfishes that have no other natural predator. The removal of these sea stars from the ecosystem lead to an explosion of his prey populations driving out most other species. Another example is offered by sea urchins, they are considered to be keystone species because they prevent by grazing the shift of a system dominated by encrusting algae to a system dominated by large fleshy erected algae. Mills et al. (1993) criticized this concept and considered that the term keystone species was widely used but too little undefined and not specific enough. One of

the main criticism made by these authors was that the keyword does not take enough into account the relationship between species and food webs. Faced with these criticisms, Paine (1995) refines the definition of a keystone species as "*a species whose impact on the other species of ecosystem is much larger than expected in terms of biomass and the abundance of this species*". However the most common accepted redefinition of the keystone species is that provided by Power et al. (1996) as *"one whose impact on its community is large, and disproportionately large relative to its abundance"*. Furthermore an exhaustive review on the concept of keystone species within the community is furnished by Piraino et al. (2002).

I.4.2 The engineering species

In 1994, Jones and other authors defined the engineering species as "*organisms that directly or indirectly modulate the availability of resources to other species by causing physical state changes in biotic or abiotic materials*". As a result, ecosystem engineers are important for maintaining the health and stability of the environment where they live. Examples of engineering species are the trees of the terrestrial forests or the corals of the marine reefs that physically transform the environment by their erected structure. The marine macrophytes such as the seagrass *Posidonia oceanica* and the seaweed belts of kelps and fucoids build real marine forests and are also considered to be engineering species. These erected organisms influence the light, the rate of sedimentation, control productivity and nutrient cycling in temperate rocky reefs providing habitat for many other species of algae and animals and greatly modify the colonized environment promoting biological diversity (Schiel and Foster 2006). Moreover the marine seaweeds and seagrasses are considered the most important benthic primary producers along the coasts all over the world (Mann 1973) (Figure **I-2**). Some of the most important engineering or foundation species in marine ecosystems as fucoids or kelps seaweeds are in decline all over the world mostly due to the combined effects of multiple local anthropic and global climatic stressors (Airoldi and Beck 2007, Lotze et al. 2011, Strain et al. 2015).

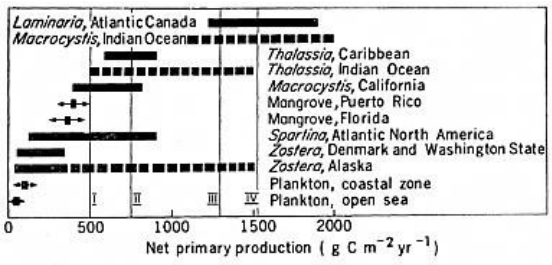


Figure I-2. The productivity of different marine macrophytes, compared with some terrestrial communities (I: medium-aged oak-pine forest, New York; II: young pine plantation, England; III: mature rain forest, Puerto Rico; IV: intensively managed system, United States). Calculated as kilocalories x 0.1. Broken lines are estimates based on biomass data. Source (Odum 1971)

I.5 The choice of the model *Cystoseira*

In the Mediterranean Sea, canopy forming algae of order Fucales are the dominant ones establishing structurally complex and diversified assemblages and functioning as engineering species (Schiel and Foster 2006). The species of the genus *Cystoseira* together with *Sargassum* are the main representatives of the order Fucales in the Mediterranean Sea.

These algae are sensitive to a variety of environmental stressors, as a consequence they are used in ecological status assessment as coastal water indicator according to the Water Framework Directive (WFD 2000/60/EU, Jncc.defra.gov.uk, 2010) (Ballesteros et al. 2007). Five species have been included in the Annex II of the Barcelona Convention and in the Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitat (known as the Bern Convention) and thus they deserve protection at a Mediterranean scale.

The genus *Cystoseira*, show a very surprising morphological plasticity both among and within the species. This morphologic variability is mainly affected by abiotic factors as water depth, hydrodynamic features, seasonality, water temperature, moreover for the Mediterranean species is not uncommon to find individuals with intermediate characteristics that are impossible to identify unambiguously. As a consequence their taxonomic identity is often the aim of the discussion even among expert phycologists.

In the last decades, the disappearance of *Cystoseira* species has been recorded in wide geographical area along the temperate rocky coasts of the Mediterranean Sea, because of cumulative impacts: habitat destruction, eutrophication, overgrazing by sea urchins, outcompetition by mussels, coastal aquaculture, invasive species, human trampling and chemical pollution are to be considered as the major threats (Thibaut et al. 2005, Mangialajo et al. 2008a, Buia et al. 2013b, Grech et al. 2015, Thibaut et al. 2015). These impacts act over time and in unison, with a possible synergistic effect on the species, the ecosystems and their ability to sustain the biodiversity. One of the most clear effect is the replacement of the sensitive species with the most stress-tolerant and opportunistic one involving a simplification of the architectural complexity of the communities (Arévalo et al. 2007).

Cystoseira species are also disappearing from the Gulf of Naples mostly due to the fragmentation and the loss of natural habitat of colonization of these algae (Grech et al. 2015). The importance of these species in structuring habitat, and their loss on the counterpart make necessary an assessment of their diversity at species, genetic and ecosystem level. Nevertheless the phenotypic plasticity of these species makes it difficult to distinguish the species from subspecies, morphotypes, varieties and ecotypes and as a consequence to assess which one is being lost. An integrated approach taking into account both morphological and genetic features could be a fundamental strategy to detect the

consequences of the loss of these species at ecosystem level and to provide tools for the restoration and coastal management.

I.5.1 Taxonomy

Empire	Eukaryota
Kingdom	Chromista
Phylum	Ochrophyta
Class	Phaeophyceae
Order	Fucales
Family	Cystoseiraceae
Genus	*Cystoseira*

I.5.2 Morphology

The species of the genus *Cystoseira* have been described by Agardh (1820) as arborescent algae (except for *Cystoseira dubia*), often of big size up to 1 meter (Gómez Garreta et al. 2001) having a single primary axis (cauloid) or many primary axes (caespitose species) (Figure I-3).

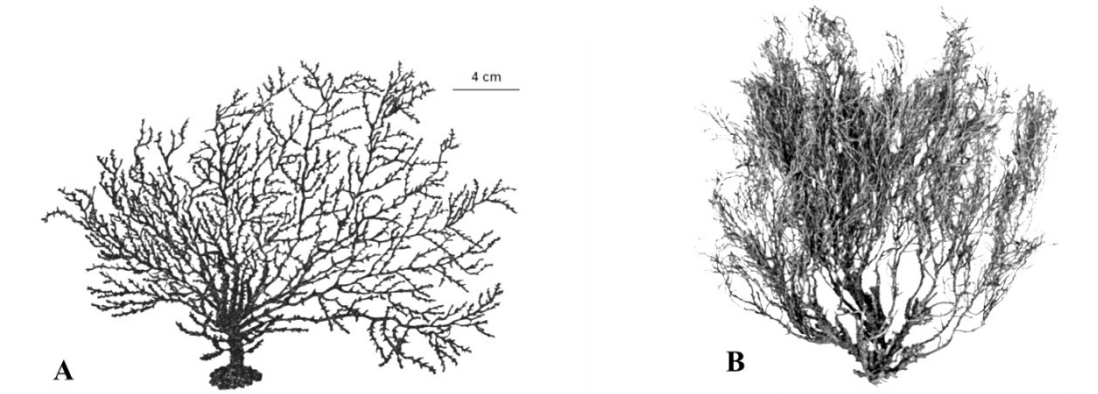


Figure I-3. Morphology of thallus. A: *Cystoseira algeriensis*, single cauloid (not caespitose algae), B: *Cystoseira brachycarpa*, multiple cauloids (caespitose algae). Source Cormaci et al. (2012)

The axis is fixed to the substratum (except in *Cystoseira barbata* f. *aurantia*) by means of a conical disc or aptery. In the species with a prostrated primary axis (stolon) this also serves as the anchoring structure. Usually the primary axes are cylindrical, simple or branched with smooth, spiny, protruding or sunken apex. The primary axis could be smooth or provided

with protuberances or scars left by the old branches or could be provided with tophules (enlargement of certain portion of the thallus). The abundant secondary branches give to these algae a bushy appearance. The branches could be radial or distichous and having or not an iridescence. Some species are provided with air vesicles (aerocysts) that could be ovoid or elliptical-elongated, isolated or in series arisen from branches expansion. On higher-order branches are frequent hairy crypts (cryptostomata). The reproductive structures or receptacles are in the terminal end of higher-order branches (Figure I-4).

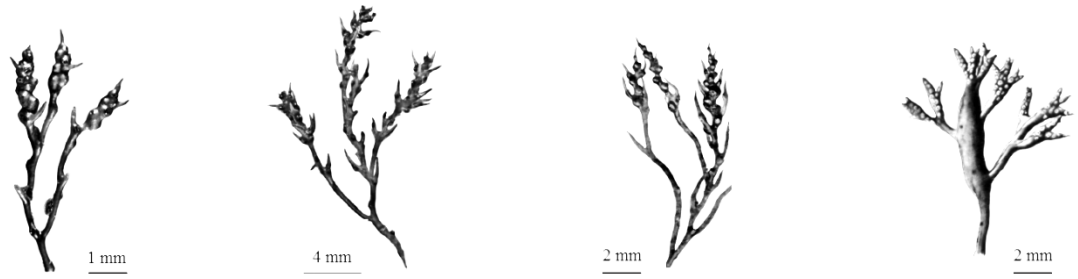


Figure I-4. Different types of receptacles. Source Cormaci et al. (2012)

The main features used to determine the species according to Gómez Garreta et al. (2001), Cormaci et al. (2012) and Taşkin et al. (2012) are:

- Free / attached plants (*Cystoseira barbata* f. / *Cystoseira compressa*)
- Caespitose / single primary axis (Figure I-3) (*Cystoseira brachycarpa* / *Cystoseira algeriensis*)
- Presence / absence of tophules (*Cystoseira zosteroides* / *Cystoseira brachycarpa*)
- Presence / absence of aerocysts (*Cystoseira usneoides* / *Cystoseira algeriensis*)
- Iridescence (*Cystoseira amentacea*, *Cystoseira mediterranea*, *Cystoseira elegans*)
- Smooth or spiny apex (*Cystoseira barbata* / *Cystoseira crinita*)
- Receptacles position on the branches

I.5.3 Life cycle

Cystoseira species reproduce sexually through a diplobiontic monogenetic life cycle, the haploid phase being only represented by gametes. Fertilization is external (Graham and Wilcox 2000). Gametangia are produced within sunken chambers (conceptacles) that develop as the diploid plants mature (Figure I-5). Eggs and bi-flagellated sperm are extruded in mucilage through the ostiole, a pore in the conceptacle, often still contained within their

respective gametangia. The fertilized eggs begin the development on the outside of receptacles (Chapman 1995). The microscopic zygotes and young juveniles take up to several months to develop to macroscopic size under optimal conditions (Schiel and Foster 2006).

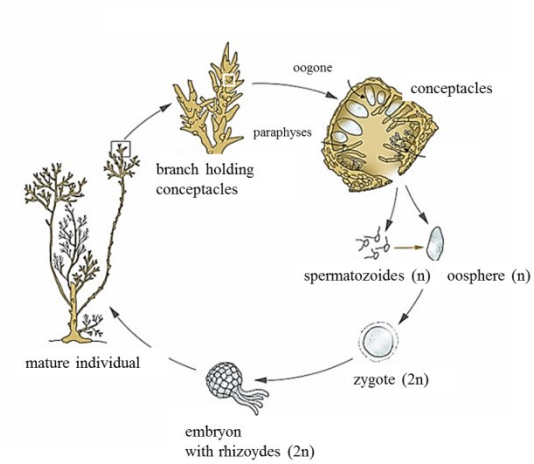


Figure I-5. Life cycle of the genus *Cystoseira*. Source Gómez Garreta et al. (2001), modified.

I.5.4 Phylogeny

In a recent work, Draisma et al. (2010) dealt with the complex taxonomy of the genus *Cystoseira*. They made their observation on the molecular data from several specimens collected globally at different localities. The results of this work stated that the examined species of *Cystoseira* are divided into six distinct clades, each identifying a distinct genus (Figure I-6). On this basis authors referred the Indo- Pacific species (clade: *Cystoseira*-1) to the genus *Sirophysalis* Kützing, the Indian species (clade: *Cystoseira*-2) to the genus *Polycladia* Montagne and the Pacific ones (clade: *Cystoseira*-3) to the genus *Stephanocystis* Trevisan. Both Atlantic European and Mediterranean species splitted into other three clades: clade *Cystoseira*-4 (that should be maintain the name *Cystoseira*) and clades *Cystoseira*-5 and *Cystoseira*-6, for which authors delayed the formal proposal of new genera depending on obtaining further anatomical, morphological and reproductive data to better characterize them.

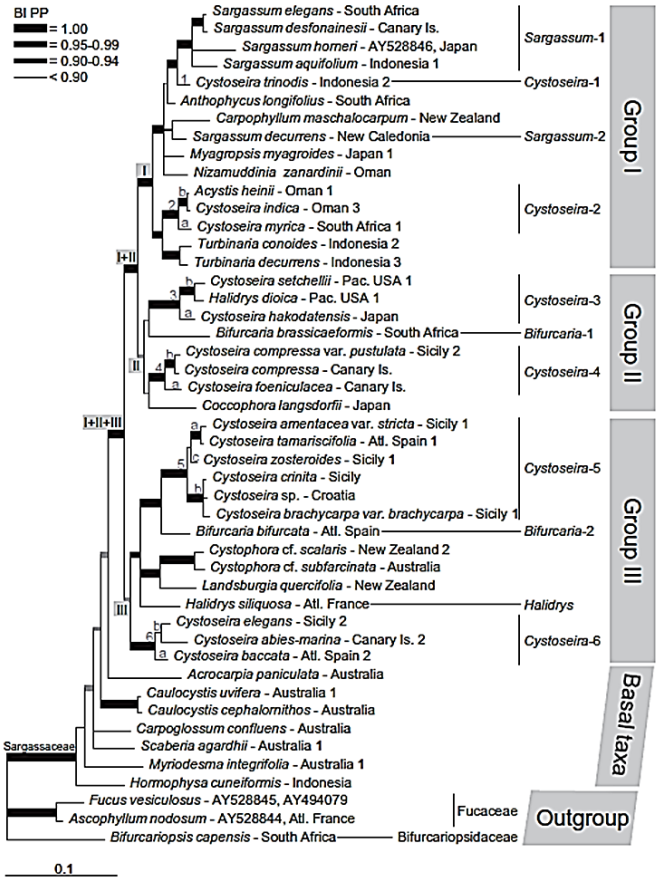


Figure I-6. Bayesian inference majority-rule consensus tree based on plastidial psbA gene and mitochondrial mt23S DNA combined sequence data as described by Draisma et al. (2010).

I.5.5 Distribution and habitat

The species of the genus *Cystoseira* are geographically widespread distributed, however their core of diversity is the Mediterranean Sea, where most of the species are endemic (Table I-2). About 42 *Cystoseira* species are currently recognized (www.algaebase.org, last accessed January, 2017) (Guiry 2017). These algae usually dominate unpolluted rocky habitat from the intertidal shore to the upper circalittoral zone (Giaccone and Bruni 1973b, Ballesteros 1992, Giaccone et al. 1994, Cormaci et al. 2012). The hydrodynamism is one of the main factor that determines the distribution of these species.

Most of the species are considered to be stenotopic since their distribution is confined to relatively few habitats and they cannot tolerate wide environmental variations. The intertidal rocky shores with elevated hydrodynamic feature are mostly characterized by the three vicariant species *C. amentacea*, *C. mediterranea* and *C. tamariscifolia*. *C. compressa* is distributed along the intertidal shore of sheltered zones with a low hydrodynamism. In the

upper infralittoral shore between about 3 and 6 meters depth are more frequent the species *C. brachycarpa* and *C. crinita*. The following second bathymetric infralittoral zone included between 8-9 meters up to 15 meters is characterized by *C. sauvaugeauana* and *C. foeniculacea* f. *tenuiramosa*, deeper over the 15 meters are found belts of *C. spinosa*. In the upper limit of the circalittoral zone is possible to find *C. dubia* assemblages especially in those habitat characterized by weak current regime and high sedimentation rate, assemblages of *C. zosteroides* are found in habitat with monodirectional currents flowing zone and in absence of sedimentation. Finally *C. usneoides* can be found at strong current regime zone with a constant water temperature. The other species of *Cystoseira* are distributed all over the Mediterranean Sea in habitat subjected to environmental variations that inhibit the development of the previously cited stenotopic species.

I.5.6 The importance of Cystoseira as habitat forming species

Cystoseira species are long-living and very productive macroalgae with a complex tri-dimensional structure providing habitat, food, shelter and nursery for a wide variety of species supporting therefore a high biodiversity (Bulleri et al. 2002, Mangialajo et al. 2008a, Vergés et al. 2009).

The value of *Cystoseira* associations as a nursery for fish (Orlando-Bonaca and Lipej 2005, Lipej et al. 2009, Riccato et al. 2009, Vergés et al. 2009, Cheminée et al. 2013) as well as the importance in structuring the invertebrate communities (Milazzo et al. 2000, Chemello and Milazzo 2002, Fraschetti et al. 2002, Gozler et al. 2010, Urra et al. 2013) has been investigated in different areas of the Mediterranean Sea. However there is a gap of knowledge regarding most sites of the Tyrrhenian Sea, among them the Gulf of Naples, where invertebrate fauna associated with canopy-forming algae of the genus *Cystoseira* has never been investigated. Amongst the invertebrate fauna inhabiting *Cystoseira* assemblages, the molluscs are one of the best represented and dominant taxa, moreover they are considered an important food source for the higher trophic levels.

Table I-2. List of the current taxonomically accepted entities of the genus *Cystoseira* with the authors and heterotypic synonyms associated with each species. * indicates the species endemic of the Mediterranean sea; var: variety; subsp: subspecies; f: formae. (Source Algaebase, www.algaebase.org, last accessed January 2017)

Species	**Var/ f / subsp**	**Author**	**Heterotypic Synonym(s)**
Cystoseira abies-marina		(S.G.Gmelin) C.Agardh 1820	*Phyllacantha moniliformis* Kützing 1843
Cystoseira abrotanifolia	var. *macrocarpa*	(Kützing) De Toni 1895	
Cystoseira adriatica	f. *reducta*	(Ercegovic) Giaccone in Giaccone & Bruni 1973	
Cystoseira algeriensis *		Feldmann 1945	
Cystoseira amentacea *		(C.Agardh) Bory 1832	*C. stricta* var. *amentacea* (Bory) Giaccone; *C. spicata* subsp. *elegans* Ercegovic 1952
Cystoseira amentacea	var. *stricta*	Montagne 1846	*C. spicata* Ercegovic 1952; *C. spicata* subsp. *crassa* Ercegovic 1952; *C. stricta* var. *spicata* (Ercegovic) Giaccone 1973; *C. amentacea* var. *spicata* (Ercegovic) G.Giaccone 1992
Cystoseira baccata		(S.G.Gmelin) P.C.Silva	*Fucus abrotanoides* S.G.Gmelin 1768; *F. fibrosus* Hudson 1778; *C. fibrosa* (Hudson) C.Agardh 1820; *C. thesiophylla* Duby 1830; *Phyllacantha fibrosa* (S.G.Gmelin) Kützing 1843; *P. thesiophylla* (Duby) Kützing 1860
Cystoseira barbata *		(Stackhouse) C.Agardh 1820	*Fucus barbatus* Goodenough & Woodward 1797; *C. hoppei* C.Agardh 1820; *C. barbata* var. *hoppei* (C.Agardh) J.Agardh 1842; *C. barbata* f. *hoppei* (C.Agardh) Woronichin 1908
Cystoseira barbata *	f. *aurantia*	(Kützing) Giaccone in Amico et al. 1985	*C. concatenata* f. *repens* A.D.Zinova & Kalugina; *C. barbata* f. *repens* A.D.Zinova & Kalugina 1974
Cystoseira barbata	f. *flaccida*	(Kützing) Woronichin 1908	
Cystoseira barbatula *		Kützing 1860	*C. graeca* Schiffner ex Gerloff & Nizamuddin 1975

Cystoseira bosphorica		Sauvageau 1912	
Cystoseira brachycarpa *		J.Agardh 1896	*C. balearica* Sauvageau 1912; *C. brachycarpa* var. *balearica* (Sauvageau) Giaccone 1992
Cystoseira brachycarpa *	var. *claudiae*	Giaccone in Ribera et al. 1992	
Cystoseira compressa		(Esper) Gerloff & Nizamuddin 1975	*C. filicina* Bory; *C. abrotanifolia* f. *fimbriata* Sauvageau; *C. fimbriata* Bory 1832; *C. compressa* subsp. *rosetta* Ercegovic 1952; *C. compressa* f. *rosetta* (Ercegovic) M.Cormaci, G.Furnari, G.Giaccone, B.Scammacca & D.Serio 1992; *Fucus fimbriatus* Desfontaines 1799
Cystoseira compressa *	f. *plana*	(Ercegovic) Cormaci, G.Furnari, Giaccone, Scammanca & D.Serio 1992	
Cystoseira compressa	subsp. *pustulata*	(Ercegovic) Verlaque in Thibaut et al. 2015	*C. planiramea* Schiffner ex Gerloff & Nizamuddin 1975; *C. epiphytica* Schiffner ex Gerloff & Nizamuddin 1976; *C. compressa* var. *pustulata* Ercegovic ex Verlaque 1988
Cystoseira corniculata		(Turner) Zanardini 1841	*C. corniculata* subsp. *laxior* Ercegovic 1952; *C. corniculata* var. *laxior* (Ercegovic) Antolić & Span 2010
Cystoseira corniculata	subsp. *divergens*	Ercegovic 1952	
Cystoseira corniculata	f. *imperfecta*	Ercegovic 1952	
Cystoseira crinita		Duby 1830	*Fucus crinitus* Desfontaines 1799
Cystoseira crinita	f. *semispinosa*	Ercegovic 1952	
Cystoseira crinitophylla *		Ercegovic 1952	
Cystoseira dubia *		Valiante 1883	*C. fucoides* Ercegovic 1952
Cystoseira elegans *		Sauvageau 1912	
Cystoseira ericoides	var. *gibraltica*	Sauvageau	

Cystoseira fimbriata	var. *pustulata*	Ercegovic	
Cystoseira foeniculacea *		(Linnaeus) Greville 1830	*Phyllacantha concatenata* (Linnaeus) Kützing; *Fucus concatenatus* Linnaeus 1753; *F. abrotanifolius* Linnaeus 1753; *F. barbatus* Linnaeus 1753; *F. discors* Linnaeus 1767 *C. concatenata* (Linnaeus) C.Agardh 1820; *C. abrotanifolia* (Linnaeus) C.Agardh 1820; *C. discors* (Linnaeus) C.Agardh 1828; *C. ercegovicii* Giaccone 1973
Cystoseira foeniculacea	f. *dubia*	(Ercegovic) Bouafif, Verlaque & Langar	
Cystoseira foeniculacea *	f. *latiramosa*	(Ercegovic) A.Gómez Garreta, M.C.Barceló, M.A.Ribera & J.R.Lluch 2001	*C. discors* subsp. *latiramosa* Ercegovic 1952; *C. ercegovicii* f. *latiramosa* (Ercegovic) Giaccone 1985; *C. schiffneri* f. *latiramosa* (Ercegovic) Giaccone 1992
Cystoseira foeniculacea *	f. *tenuiramosa*	(Ercegovic) A.Gómez Garreta, M.C.Barceló, M.A.Ribera & J.Rull Lluch 2001	
Cystoseira funkii *		Schiffner ex Gerloff & Nizamuddin 1976	
Cystoseira helvetica		Heer 1877	
Cystoseira humilis		Schousboe ex Kützing 1860	*C. barbata* var. *pumila* Montagne 1841; *C. pumila* Kützing 1860; *C. canariensis* Sauvageau 1912
Cystoseira humilis	var. *myriophylloides*	(Sauvageau) J.H.Price & D.M.John in J.H.Price, D.M.John & G.W.Lawson 1978	
Cystoseira hyblaea *		Giaccone 1985	
Cystoseira hypocarpa		Kützing 1854	
Cystoseira macrocarpa		Kützing 1854	

Cystoseira mauritanica		Sauvageau in Hariot 1911	*C. selaginoides* var. *gibraltarica* Sauvageau 1920; *C. sauvageauana* var. *gibraltarica* (Sauvageau) Hamel 1939; *C. gibraltarica* (Sauvageau) P.Dangeard 1949
Cystoseira mediterranea *		Sauvageau 1912	*C. mediterranea* var. *valiantei* Sauvageau 1912
Cystoseira melanothrix		(Kützing) Piccone 1884	
Cystoseira montagnei *		J.Agardh 1842	*C. granulata* var. *turneri* Montagne 1838
Cystoseira myrica	var. *occidentalis*	J.Agardh	
Cystoseira nodicaulis		(Withering) M.Roberts 1967	*Fucus mucronatus* Turner
Cystoseira occidentalis		Gardner 1923	
Cystoseira pelagosae *		Ercegovic 1952	
Cystoseira pycnoclada		Schiffner ex Gerloff & Nizamuddin 1976	
Cystoseira rayssiae *		Ramon 2000	
Cystoseira sauvageauana *		Hamel 1939	*C. selaginoides* var. *polyoedematis* Sauvageau 1912; *C. sauvageauana* var. *polyoedematis* (Sauvageau) Hamel 1939; *C. sicula* Schiffner ex Gerloff & Nizamuddin 1976
Cystoseira schiffneri		Hamel 1939	C. acanthophora Schiffner 1926
Cystoseira sedoides *		(Desfontaines) C.Agardh 1820	
Cystoseira selaginoides		Naccari 1828	
Cystoseira senegalensis		P.A.Dangeard 1938	
Cystoseira sonderi		(Kützing) Piccone 1886	
Cystoseira spicigera		C.Agardh 1820	
Cystoseira spinosa *		Sauvageau 1912	*Fucus erica-marina* S.G.Gmelin *C. erica-marina* (S.G.Gmelin) Naccari 1828; *C. adriatica* Sauvageau 1912

Cystoseira spinosa *	var. *compressa*	(Ercegovic) Cormaci, G.Furnari, Giaccone, Scammacca & D.Serio 1992	*C. platyramosa* Ercegovic 1952; *C. adriatica* subsp. *intermedia* Ercegovic 1952
Cystoseira spinosa *	var. *tenuior*	(Ercegovic) M.Cormaci, G.Furnari, G.Giaccone, B.Scammacca, & D.Serio 1992	*C. jabukae* Ercegovic 1952; *C. jabukae* subsp. *tenuissima* Ercegovic 1952; *C. adriatica* subsp. *reducta* Ercegovic 1952; *C. rechingeri* Schiffner ex Gerloff & Nizamuddin 1975; *C. gerloffii* Nizamuddin 1978; *C. jabukae* f. *tenuissima* (Ercegovic) M.Cormaci, G.Furnari, G.Giaccone, B.Scammacca, & D.Serio 1992
Cystoseira squarrosa *		De Notaris 1841	
Cystoseira susanensis *		Nizamuddin 1985	
Cystoseira tamariscifolia		(Hudson) Papenfuss 1950	*Fucus selaginoides* Linnaeus 1759; *F. ericoides* Linnaeus 1763 *C. ericoides* (Linnaeus) C.Agardh 1820; *C. selaginoides* (Linnaeus) Bory 1832; *C. ericoides* var. *laevis* P.J.L.Dangeard 1949; *C. ericoides* var. *divaricata* P.J.L.Dangeard 1949
Cystoseira thysigera		Postels & Ruprecht 1840	
Cystoseira usneoides		(Linnaeus) M.Roberts 1968	*Fucus granulatus* Linnaeus 1763
Cystoseira wildpretii		Nizamuddin 1995	
Cystoseira zosteroides		(Turner) C.Agardh 1821	*Carpodesmia zosteroides* (C.Agardh) Greville 1830 *C. opuntioides* (Bory ex Montagne) Kützing 1860; *C. opuntioides* Bory ex Montagne 1846 *Phyllacantha opuntioides* (Bory ex Montagne) Kützing 1849

I.6 Molluscs

Molluscs are one of the most diverse phylum of invertebrate animals on the planet, with at least 85.000 recognized living species. Most of them are marine extending from the intertidal to the deepest ocean, many also live in the freshwater and terrestrial habitats. Despite their amazing diversity, all the molluscs share some unique features that define their body plan.
The body is composed by a head, a foot and a visceral mass covered by the mantle.
They are often provided with a hard exoskeleton, the shell that is secreted by the mantle. The buccal cavity contains a radula, a ribbon of teeth supported by a muscular structure generally used for feeding. The ventral foot has adapted to various purposes in the different classes. The circulatory system is open, the blood contains the hemocyanin, the respiratory pigment. Typically, at least in the more primitive members of each group, there is one or more pairs of gills (ctenidia) that lie in a posterior cavity (the pallial cavity) or in a posterolateral groove surrounding the foot. The pallial cavity is the space into which the kidneys, gonads, and anus open. Mollusca reproduce sexually, the most common kind of fertilization is external. The development could be direct or not with a larval stage. The different feeding habits appear to have had an important influence on molluscs evolution. Most molluscs are herbivorous grazing on algae or filter feeders that feed by filtering suspended matter and food particles from the water column, the most evolved ones are primarily active predators. Because of the great range of anatomical diversity among molluscs, usually the most common features are described by a hypothetical generalized mollusc (Figure I-7).

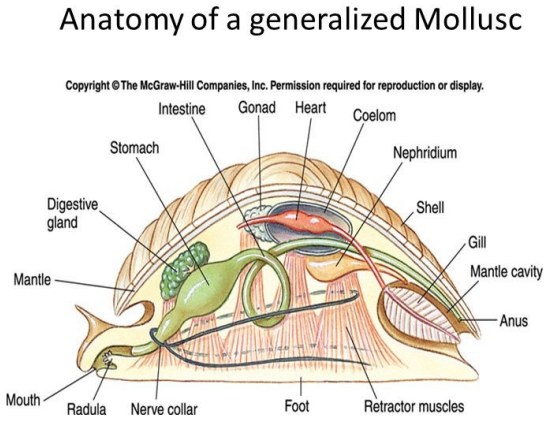


Figure I-7. Anatomy of a generalized mollusc

The molluscs systematic is still in flux, the number of the classes is still under discussion. Commonly is possible to identify eight classes: Caudofoveata, Aplacophora, Polyplacophora, Monoplacophora, Gastropoda, Cephalopoda, Bivalvia and Scaphopoda. Following a brief description of the main classes of molluscs associated with *Cystoseira*.

I.6.1 Bivalvia

Bivalvia include clams, oysters, mussels, scallops and many other families that live in saltwater or freshwater. Bivalvia are easily recognizable by a calcareous shell consisting of two hinged parts called valves whose edge in most cases is equipped with teeth. The valves are held together at the hinge by a flexible ligament. Near the hinge is the umbo, a rounded protuberance. The hinge line is the dorsal region of the shell and the lower curved margin is the ventral region. In the front of the shell are located the byssus (when present) and foot, in the posterior there are the siphons. The main muscular systems in bivalves are the posterior and anterior adductor muscles that connect the two valves and contract to close the shell. These muscles work in opposition to the ligament which tends to pull the valves apart. Bivalvia have no head, the nervous system consist of a nerve network and a series of paired ganglia. They also lack a radula, most bivalves are filter feeders using their gills to capture the particles of food in the water. The pallial cavity surrounds the whole body (Figure I-8).
The sexes are usually separate, fertilization is usually external, the fertilized eggs hatch into trocophore larvae in few hours or days before. These later develop into veliger larvae which settle on the substrate and undergo metamorphosis into juvenile. Most of the bivalve larvae feed on phytoplankton, other are lecithotrophic depending on nutrients stored in the yolk of the eggs. They can burrow into the sediment or lie on the sea floor or attach to the rocks or other hard surfaces, some such as the scallops can even swim snapping their shell.

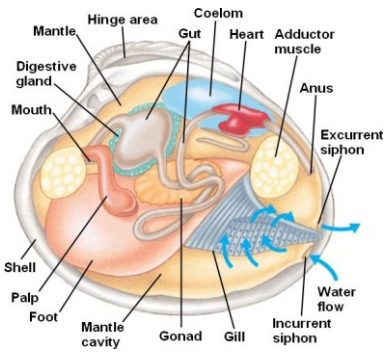


Figure I-8. Bivalve anatomy

I.6.2 Gastropoda

Gastropoda include snails, slugs, limpets, sea slugs. Most of them are marine but many live in freshwater or on land. Most Gastropoda members are characterized by a single often coiled or spiraled shell, although this is lost in some slug groups (Figure I-9). Some species have a kind of lid to close the shell called operculum. Snails are characterized by an anatomical process known as torsion that imply a 180° rotation to one side during the development. The gastropods have a well-defined head with two or four sensory tentacles sustaining from simple to more complex eyes. The radula is usually adapted to the food that a species eats. Many marine gastropods are burrowers and the mantle edge is extended anteriorly to form an inhalant siphon. The diet of gastropods differs according to the group considered. Marine gastropods include herbivores that scrape algae off the rocks, detritus feeders, carnivores, scavengers and parasites. Apart from opisthobranchs, marine gastropods have separate sexes, fertilization is external or internal according to the species. Some gastropod have a trocophore and/or veliger larval stadium.

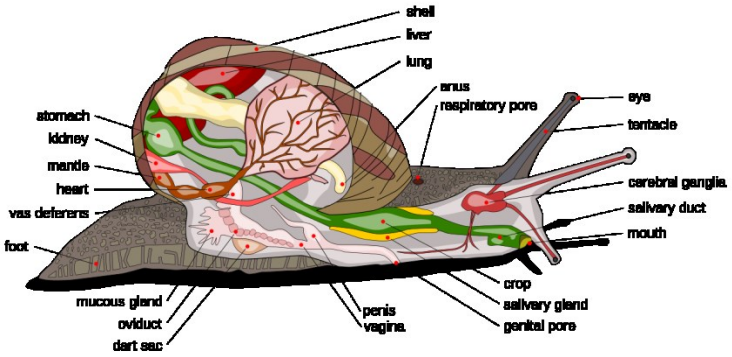


Figure I-9. Gastropoda anatomy

I.6.3 Polyplacophora

Polyplacophora include chitons. Chitons are exclusively marine, living on the hard surfaces. All the chitons bear a dorsal shell composed of eight aragonite shell plates or valves. The shell plates are surrounded by a border known as girdle. The girdle may be ornamented with spicules, bristles, hairy tufts, spikes, or snake-like scales which, like the shell plates, are mineralized with aragonite(Figure I-10). Most of the body of Polyplacophora is composed by a snail-like foot, they lack a clearly demarcated head. The mantle cavity consists of a narrow channel on each side lying between the body and the girdle. Multiple gills hang down into the mantle cavity, each consisting of a central axis with a number of flattened filaments

through which oxygen can be absorbed. The mouth is provided with a radula used to scrape microscopic algae off the substratum.

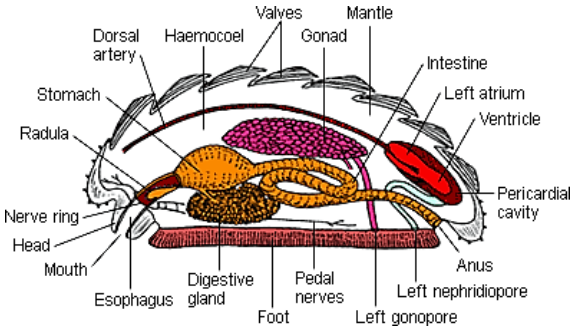


Figure I-10. Polyplacophora anatomy

I.7 Background of research

The use of the historical data is an important tool to detect and understand recent changes that may occur in marine ecosystems. In the Gulf of Naples a recent study to outline historical changes in macroalgal diversity highlighted a drastic decrease of *Cystoseira* species in the intertidal zones. The decline is more evident in the Bay of Naples, the Bay of Pozzuoli, the northern part of Ischia Island and large parts of Peninsula Sorrentina where only single scattered individuals of two *Cystoseira* species, *Cystoseira amentacea* and *Cystoseira compressa* are still present (Buia et al. 2013a, Grech et al. 2015). The influence of coastal development on the decline of *Cystoseira* species in the Gulf has been analyzed (Grech et al. 2015). Results from this study testify that the loss of species corresponds with the higher development of artificial infrastructure.

The importance of these algal species in structuring complex and diversified habitat and their disappearance in the Gulf of Naples on the other part, make more urgent than ever an investigation of the extent of diversity of these assemblages as well as of the associated fauna biodiversity.

I.8 Aims of the thesis and structure

The present thesis has been developed as a series of manuscripts for publications and thus each chapter represents a stand-alone manuscript.

The analysis of biodiversity at species, genetic and ecological level of the engineering species is an important tool for their effective protection and management.

In order to better assess the long-term consequences of the current process of *Cystoseira* population fragmentation in the Gulf of Naples at species, population and community level, the main aims of the present Ph.D. thesis can be divided in two parts that correspond with two chapters.

At species and population level (Chapter II):

1. assess the genetic characterization to solve the taxonomic ambiguity on some species through the sequence analysis of amplified psbA gene
2. analyze the genetic variability and pattern of connectivity among populations by means of sequence analysis of amplified ITS regions and microsatellites and test for the first time a next generation sequencing approach through RADSeq analysis (Restriction-site Associated DNA Sequencing) that allows to detect the Single Nucleotide Polymorphisms (SNPs) in the whole genome

At community level (Chapter III):

3. characterize molluscs assemblage structure associated with three *Cystoseira* associations along the coasts of Ischia Island where continuous belt of these algae still persist
4. determine whether, at a small spatial scale of observation, the three algal species at different sampling sites support a different pattern of associated diversity.

I.9 The study area

The Gulf of Naples (GoN) is an approximately rectangular semi-enclosed basin located over the continental shelf in the south-eastern Tyrrhenian Sea (Western Mediterranean Sea) (Uttieri et al. 2011). It spans from 40°50'N, 40°32'N to 13°52'E, 14°28'E, with an extension of approximately 900 Km^2 and an average depth of 170 m (Carrada et al. 1980).

It is bordered by the islands of Ischia and Procida and Campi Flegrei in the northern part, and by the Island of Capri and the Sorrento peninsula in the southern part (Figure I-11). The exchanges with the southern Tyrrhenian Sea occur through two main openings called Bocca Grande and Bocca Piccola. Bocca Grande between Ischia and Capri Islands, it is characterized by the presence of two canyons, Magnaghi and Dohrn reaching the maximum depths around 800 m, both canyons control the vertical fluxes acting as a channel for the transportation of sediment from the shelf to the slope (Cianelli et al. 2011). Bocca Piccola separates Capri from the Sorrento peninsula through a 74 m deep sill which slopes down to the 1000 m (Aiello et al. 2001).

The communication with the neighboring Gulf of Gaeta (in the north) and the Gulf of Salerno (in the south) are respectively guaranteed by the Ischia and Procida channels and by the Bocca Piccola (Uttieri et al. 2011). In the GoN can be identified three marginal sub-basins: the Bay of Pozzuoli in the northern part, the Bay of Naples in the northeastern sector is the coastal area flowing through the city of Naples and the Gulf of Castellammare in the southeastern part of the basin, in front of Castellammare di Stabia and the neighboring areas and receiving the freshwater input from the Sarno river (Cianelli et al. 2011). The morphology of the coasts varies from north to south: the sandy coasts present in the northern and eastern part of the basin are replaced by rapidly declining calcareous cliffs in the south (Uttieri et al. 2011).

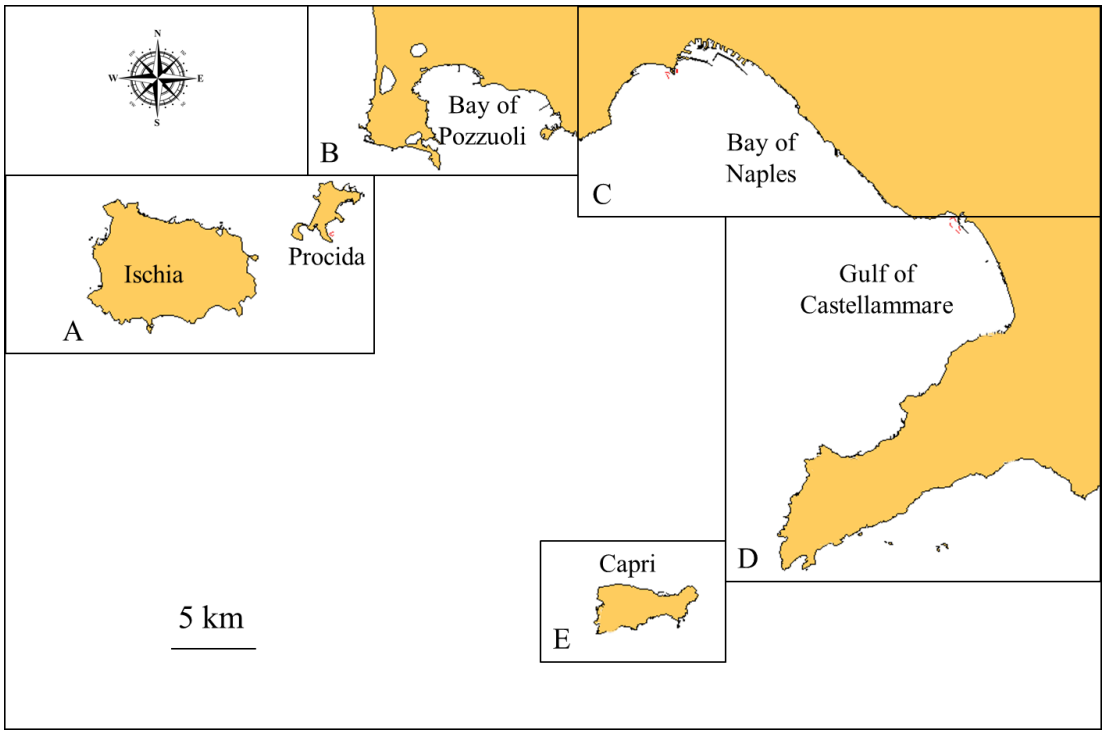


Figure I-11. Gulf of Naples with the flegrean Island of Ischia and Procida (A), the three marginal sub-basins: bay of Pozzuoli (B), bay of Naples (C), Gulf od Castellammare (D) and Capri Island (E).

The study area is also characterized by peculiar orographic aspects influencing wind and sea dynamics. The Vesuvius volcano (1.281 m) and the hills surrounding the city of Naples (with altitudes up to 450 m) can shelter northeasterly winds blowing over the basin mostly in winter, creating jet currents responsible for the rapid water exchanges (Cianelli et al. 2011). Mountains are also present in the southern edge of the GoN (Lattari Mountains; Mount Faito, 1.131 m).

The surface circulation in the GoN is the result of driving factors acting over different spatio-temporal scales and of their interaction with the complex bottom topography and orography of the basin. Such factors can be differentiated as local and remote (Gravili et al. 2001), concerning the first one, the wind is the most important factor (Menna 2007) whereas for the latter the main role is played by the circulation of the southern Tyrrhenian Sea (Gravili et al. 2001). The hydrology in the GoN presents a seasonal pattern characterized by the summer stratification of the water column determining the formation of a surface mixed layer 30-40 m thick; by contrast, the intense winter mixing involves the entire water column which is homogeneous down to 150 m (Carrada et al. 1980).

The environmental quality of the marine ecosystem in the GoN is directly influenced by human activities (Ribera d'Alcalà et al. 1989, Zingone et al. 1995, Zingone et al. 2010). The human activities range from the urban settlements to the industrial areas located on the coast and intense maritime traffic. The GoN is amongst the most densely inhabited Italian areas, and along its 195 Km of coasts approximately 30 ports and more than 300 maritime constructions are located. The Bay of Naples constantly receives the urban sewage and other eutrophising inputs from the city of Naples and the adjacent areas (Ribera d'Alcalà et al. 1989). On the other hand, the Gulf of Castellammare is affected by the runoff of the Sarno river (Zingone et al. 1995).

The GoN also hosts four marine protected areas, selected on the basis of environmental parameters as well as historical relevance. As a consequence, the maintenance and improvement of the environmental quality of the GoN is of critical importance not only for the welfare of the entire ecosystem, but also for social and economic reasons (Cianelli et al. 2011).

CHAPTER II

GENETIC VARIABILITY OF MACROALGAE OF THE GENUS *CYSTOSEIRA* IN THE GULF OF NAPLES

II.1 Introduction

The canopy-forming fucoids (Heterokonta, Phaeophyceae, Fucales) are the dominant algae along the temperate rocky coasts in pristine environment (Schiel and Foster 2006). They are considered 'foundation species' (Dayton 1972) that creates three-dimensional habitats providing shelter, food, nursery for a wide variety of associated organisms, moreover their high level of primary production support diversified functional and trophic levels (Schiel and Foster 2006). The species of the genus *Cystoseira* together with *Sargassum* are the main representatives of the order Fucales in the Mediterranean Sea where most of them are endemic, and usually dominate unpolluted rocky habitat from the upper infralittoral to the upper circalittoral zone (Giaccone and Bruni 1973b, Ballesteros 1992, Giaccone et al. 1994, Cormaci et al. 2012). Most *Cystoseira* species are stenoecious that means they have narrow environmental tolerances and thus are sensitive to a variety of environmental stressors, as a consequence they are used in the ecological status assessment as biological indicator according to the Water Framework Directive (WFD 2000/60/EU) (Serio et al. 2006, Ballesteros et al. 2007, Mangialajo et al. 2008b). Five species have been included in the Annex II of the Barcelona Convention and in the Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitat (known as the Bern Convention) and thus they deserve some protection at a Mediterranean scale. Over the last few decades the disappearance of *Cystoseira* species has been recorded in wide geographical area along the temperate rocky coasts of the Mediterranean Sea (Cormaci and Furnari 1999, Thibaut et al. 2005, Serio et al. 2006, Mangialajo et al. 2008a, Buia et al. 2013a, Grech et al. 2015) because of cumulative impacts: habitat destruction, eutrophication, overgrazing by sea urchins, competition by mussels, invasive species, human trampling and chemical pollution are considered the major threats (Thibaut et al. 2005, Airoldi and Beck 2007, Arévalo et al. 2007, Mangialajo et al. 2008a, Thibaut et al. 2015).

This trend has been also recorded in the Gulf of Naples where the recent occurrence and distribution of the genus *Cystoseira* along the upper infralittoral shore has been assessed comparing historical data (Buia et al. 2013b, Grech et al. 2015). Results have highlighted a drastic loss of *Cystoseira* species in the historical site all over the Gulf where previously them occurred and it corresponds to the highest percentage of coast transformation that leads as a direct consequence the habitat fragmentation. However assemblages of these algal species still persist in some localities along the coasts of Ischia Island in the intertidal zone although an ongoing homogenization of this genus diversity is evident (Grech et al. 2015). In the rest of the Gulf of Naples irregular belts and isolate patches of *Cystoseira* species are present (personal observation). In order to detect the consequences of *Cystoseira* loss at

species, population and ecosystem level and to provide tools for restoration and coastal management strategies, the first fundamental step is to assess the correct taxonomical identity of the species. Moreover the analysis of genetic variability is further crucial for the long-term survival and persistence of these species.

The species identification based merely on morphological features is often unreliable in the order Fucales, even when made by expert phycologists, mostly due to the polymorphic nature and phenotypic plasticity with the presence of subspecies, morphotypes, varieties, ecotypes (Coyer et al. 2006). The intraspecific variation have been attributed to different causes such as the direct responses to single or combined abiotic factors, localized mosaics of phenotypes linked to genotypes adapted to specific environmental conditions, self-fertilization, hybridization.

Amongst the Fucales, the genus *Cystoseira* is one of that needs a more urgent up-to-date reassessment from a taxonomic and systematic point of view since this genus is considered under process of active speciation (Roberts 1978). As a consequence a molecular genetic approach represents the fundamental strategy to unambiguously identify species belonging to the genus *Cystoseira* and to investigate the population genetic structure.

Since the early 1980, molecular biology has become a new important tool within the systematic of algae (Olsen 1990). Rousseau and De Reviers (1999) analyzed the molecular phylogeny of European Fucales combining the large and small subunit of ribosomal DNA. Serrão et al. (1999) studied the evolution of Fucaceae through the nuclear Internal Transcribed Spacer (ITS1 and ITS2), Cho et al. (2006) inferred phylogenetic relationship within Fucales by means of plastid photosystem I coding psaA sequences, Silberfeld et al. (2010) combined five mitochondrial and four plastidial genes to clarify relationship among brown algae. However molecular data on the genus *Cystoseira* have become available only recently (Harvey and Goff 2006, Susini 2006, Draisma et al. 2010) and have highlighted a very complex evolutionary scenario. PsbA and mt23S phylogeny inferred by Draisma et al. (2010) showed that the genus *Cystoseira* is polyphyletic.

In order to better assess the long-term consequences of the current process of *Cystoseira* population fragmentation in the Gulf of Naples at species and population level, the main aims of the present work can be resumed as follows:

a) investigate the genetic characterization to solve the taxonomic ambiguity on some species through the sequence analysis of amplified psbA gene.

The psbA is a plastidial gene codifying the thylakoid protein D1 that binds the chlorophyll molecules in the Photosystem II. The choice of this molecular marker is linked to:

- ecological and physiological importance of this gene involved in photosynthetic process
- large utilization for molecular phylogeny because it is highly conserved and little variable in the lineages.

b) analyze the genetic variability and pattern of connectivity among population by means of sequence analysis of ITS regions and microsatellites and test for the first time a next generation sequencing approach through RADSeq analysis (Restriction-site Associated DNA Sequencing).

ITS (Internal Transcribed Spacer) is a nuclear ribosomal DNA no-codifying region, placed between structural and codifying rDNA genes. The eukaryotic rDNA consists of the 18S, 5.8S, and 28S rRNA genes transcribed as a unit by RNA polymerase I. Post-transcriptional processes split the cistron, removing two internal transcribed spacers. These two spacers, are usually referred as the ITS region. ITS 1 and ITS 2 are largely used in taxonomy and molecular phylogeny because of:

- high number of repeated copies
- a lot of spontaneous harmless mutations
- the presence of preserved stretches at the beginning and the end of these sequences that makes possible the utilization of universal primers.

Microsatellites are short tandem repeats (STRs) or simple sequence repeats (SSRs) in which definite DNA motifs (usually a di-, tri-, tetra- or pentanucleotides) are repeated from 5 to 50 times. Microsatellites are distributed throughout the genome and tend to occur in non-coding regions of the DNA. The latter feature allows microsatellites to accumulate unhindered mutations over the generation producing variability which can be used for population genetic analysis. Microsatellites are useful genetic markers for different reasons:

- locus-specific
- co-dominant (heterozygosis and homozygosis are distinguishable)
- highly polymorphic
- their analysis does not require refined techniques since are PCR-based.

The RADSeq or Restriction-site Associated DNA Sequencing allows to detect the Single Nucleotide Polymorphisms (SNPs) in the whole genome by isolating RAD tags, that are the DNA sequences flanking a particular restriction site of a restriction enzyme. The RADSeq is a next-generation sequencing-based method that has been recently used in ecological, evolutionary and conservation genomics studies. It allows the detection of hundreds or thousands of polymorphic genetic markers across the genome in a single, simple and cost-effective experiment (Luikart et al. 2003, Davey and Blaxter 2010). These techniques require

high-molecular-weight genomic DNA that will be digested by one or more restriction enzymes. Consequently, specific sequencing adaptors, or double-stranded oligonucleotides are linked in order to be suitable for all the next-generation sequencing platforms. Adaptors added during the RADSeq protocols may contain barcodes, which are used to identify individual samples that are sequenced together (multiplexed) in a single genomic library.

II.2 Materials and Methods

II.2.1 Sample collection and DNA extraction

The algal specimens were collected along the coasts of the Gulf of Naples from the localities as listed in the Table II-1 according to the previously known presence of the assemblages dominated by *Cystoseira* species (Grech PhD thesis unpublished data). The individuals were collected either by snorkeling in the upper sublittoral rocky zone or by SCUBA diving (maximum depth 26 meters). At each locality from a minimum of 10 up to 30 individuals were sampled along a linear transect at least 1 meter each other to avoid collecting clones since the gametes and propagules of the fucoids algae are negatively buoyant and settle immediately after ejection (Pearson and Serrao 2006).

The species were identified in *situ* and in case of doubt a specimen was collected, and observed at the laboratory following the identification key as described by Gómez Garreta et al. (2001) and Cormaci et al. (2012).

Depending on thalli dimension, one or more branches were excised from the apical tip. The samples were preserved in individual tubes with seawater inside a cooled box up to the arrival at the laboratory. The leaves were rinsed in filtered seawater to remove epiphytes and immediately processed or frozen at -80°C or air-dried in silica gel.

DNA from 100-150 mg of tissue was extracted by modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). MagAttract® Suspension G by Qiagen was used to improve the purity of DNA and avoid contamination by viscous soluble polysaccharides and secondary compounds that inhibit down-stream enzymatic reactions (Huang et al. 2000). The detailed protocol is described in the Appendix 1

The quality and the size of the extracted genomic DNA were evaluated by 1% agarose gel electrophoresis in 0.5X TBE (5X TBE: 1.1M Tris; 900mM Borate; 25mM EDTA; pH 8.3).

Table II-1. List of species and their collection localities with geographic coordinates and habitat features. Number of specimens are recorded.

Species	Locality	Coordinates	Habitat	Nr. Samples
Cystoseira compressa	Punta Caruso, Ischia (PC)	40°45.46'N; 13°51.74'E	sublittoral (0 mt)	9
Cystoseira compressa	Scannella, Ischia (SC)	40°42.23'N; 13°51.66'E	sublittoral (0 mt)	6
Cystoseira compressa	Castello Aragonese, Ischia (CA)	40°43.84'N; 13°58.02'E	sublittoral (0 mt)	10
Cystoseira compressa	S. Pancrazio, Ischia (SP)	40°42.28'N; 13°57.06'E	sublittoral (0 mt)	10
Cystoseira compressa	Punta del Lume, Ischia (PL)	40°42.54'N; 13°57.45'E	sublittoral (0 mt)	7
Cystoseira compressa	Capo Miseno, Bacoli (BA)	40°46.68'N; 14°05.34'E	sublittoral (0 mt)	2
Cystoseira compressa	Punta dell'Arcera, Capri (CP1)	40°33.59'N; 14°11.92'E	sublittoral (0 mt)	1
Cystoseira compressa	Cala del Rio, Capri (CP2)	40°33.20'N; 14°12.01'E	sublittoral (0 mt)	1
Cystoseira compressa	Bagni Tiberio, Capri (CP3)	40°33.60'N; 14°13.54'E	sublittoral (0 mt)	1
Cystoseira compressa	Punta Masullo, Capri (CP4)	40°32.80'N; 14°15.57'E	sublittoral (0 mt)	1
Cystoseira amentacea	Punta Caruso, Ischia (PC)	40°45.46'N; 13°51.74'E	sublittoral (0 mt)	4
Cystoseira amentacea	Scannella, Ischia (SC)	40°42.23'N; 13°51.66'E	sublittoral (0 mt)	7
Cystoseira amentacea	Castello Aragonese, Ischia (CA)	40°43.84'N; 13°58.02'E	sublittoral (0 mt)	6
Cystoseira amentacea	S. Pancrazio, Ischia (SP)	40°42.28'N; 13°57.06'E	sublittoral (0 mt)	5
Cystoseira amentacea	Punta Imperatore, Ischia (PI)	40°42.66'N; 13°51.06'E	sublittoral (0 mt)	9
Cystoseira amentacea	Capo Miseno, Bacoli (BA)	40°46.68'N; 14°05.34'E	sublittoral (0 mt)	1
Cystoseira amentacea	Gaiola, Napoli (NA)	40°47.53'N; 14°11.22'E	sublittoral (0 mt)	1
Cystoseira amentacea	S. Angelo, Ischia (SA)	40°41.73'N; 13°53.49'E	sublittoral (0 mt)	7

Cystoseira amentacea	Punta dell'Arcera, Capri (CP1)	40°33.59'N; 14°11.92'E	sublittoral (0 mt)	1
Cystoseira amentacea	Cala del Rio, Capri (CP2)	40°33.20'N; 14°12.01'E	sublittoral (0 mt)	1
Cystoseira amentacea	Bagni Tiberio, Capri (CP3)	40°33.60'N; 14°13.54'E	sublittoral (0 mt)	1
Cystoseira amentacea	Punta Masullo, Capri (CP4)	40°32.80'N; 14°15.57'E	sublittoral (0 mt)	1
Cystoseira crinita	Scannella, Ischia (SC)	40°42.23'N; 13°51.66'E	tide pool (0 mt)	9
Cystoseira brachycarpa	Scoglio delle Sirene, Capri (CP5)	40°32.66'N; 14°14.09'E	tide pool (0 mt)	1
Cystoseira brachycarpa	Punta dell'Arcera, Capri (CP1)	40°33.59'N; 14°11.92'E	sublittoral (0 mt)	1
Cystoseira sauvageauana	Punta Carena, Capri (CP6)	40°32.16'N; 14°11.90'E	infralittoral (-16mt)	1
Cystoseira spinosa	Punta Carena, Capri (CP6)	40°32.16'N; 14°11.90'E	infralittoral (-27mt)	1

II.2.2 psbA and ITS PCR amplification

PCR amplification were performed in a EuroClone Thermal Cycler (Pero, MI, Italy). Primer sequences are listed in the Table II-2.

A 25 µL reaction volume containing 1 µL of 10-100 times diluted genomic DNA, 2.5 µL 10X PCR buffer (Roche), 0.5 $\mu g \cdot \mu l^{-1}$ 0.1% bovine serum albumin (BSA), 2.5 µL dNTPs (2mM each), 1.25 µL of each forward and reverse primer (10 pmol/µl), 0.25 µL Taq DNA Polymerase 5U/µL (Roche) and brought to the final volume with MilliQ water.

For psbA, an initial denaturation step for 5 min at 94°C was followed by 35 cycles of 1 min at 94°C, 40 s at 52°C, and 1 min at 72°C, with a final step of 10 min at 72°C. For ITS, an initial denaturation step for 3 min at 94°C was followed by 35 cycles of 30 s at 94°C, 30 s at 45°C, and 45 s at 72°C, with a final step of 10 min at 72°C. ITS was amplified with ITSP1/G4. If this failed, samples were amplified into two parts with ITSP1/ITSR1 and P5/G4. PCR products were screened for correct length by agarose gel electrophoresis (1.5 %, 0.5X TBE) stained with ethidium bromide and purified with GenElute™ PCR Clean-Up Kit or GenElute™ Gel Extraction Kit by SIGMA following the manufacturer's instructions. The cleaned PCR products were sequenced for both directions using amplification primers on 48 capillaries Applied Biosystems (Life Technologies) 3730 DNA Analyzer using Big Dye®-terminator chemistry at the Molecular Biology Service (SBM), Stazione Zoologica Anton Dohrn, Naples.

Table II-2. psbA and ITS PCR amplification and sequencing primers

Primer_ID	Primer Sequence (5'-3')	Gene	Size (bp)	References
psbA-F	ATGACTGCTACTTTAGAAAGACG	psbA	900	Yoon et al. (2002)
psbA-R	GCTAAATCTARWGGGAAGTTGTG	psbA	900	Yoon et al. (2002)
ITSP1-F	GGAAGGAGAAGTCGTAACAAGG	ITS1, ITS2	1300	Tai et al. (2001)
G4-R	CTTTTCCTCCGCTTATTGATATG	ITS1, ITS2	1300	Tai et al. (2001)
ITSR1-R	TTCAAAGATTCGATGATTCAC	ITS2	700	Tai et al. (2001)
P5-F	GCATCGATGAAGAACGCAG	ITS2	700	Tai et al. (2001)

II.2.3 Microsatellites PCR amplification

Eight microsatellites already described for the species *Cystoseira amentacea* (Robvieux et al. 2012) were used to test the suitability of these molecular markers within the individuals of the present study and to compare the DNA motif of repeats. These microsatellites were

tested on 3 individuals of *Cystoseira amentacea* from one population in the Gulf of Naples. The primers were the same described by Robvieux et al. (2012) listed in the Table II-3.

The PCR reaction volume was the same as described for psbA and ITS. PCR cycle was as follows: 95°C for 5 min, 30 cycles of 30 s at 95°C, 1 min 30 s at 60°C, 30 s at 72°C and a final extension of 7 min at 72°C. Direct sequencing of microsatellites PCR products resulted in unresolved chromatograms often with multiple peaks, thus purified PCR products were cloned using the TOPO TA Cloning® Kit. PCR-amplified products were ligated to the pCR™4-TOPO® TA Vector by ThermoFisher Scientific following the manufacturer's instructions and then transformed in One Shot TOP10® Chemically Competent *E. coli*.

The detailed protocol of cloning and transformation is described in the Appendix 2.

Ten to twenty colonies were screened to confirm the presence of the insert and colony PCR with specific vector primers was performed. Plasmid DNA was isolated from recombinant *E. coli* cells through GenElute Plasmid Miniprep Kit by Sigma. The products were sequenced over both strands with vector specific primers using the same tools as described for psbA and ITS.

Table II-3. Microsatellites primers from Robvieux et al. (2012)

Genbank accession number	Primer_ID	Primer sequence (5'-3')	Product size (bp)
JN181245	Microsat 1-F	TGTGTGTGTGCGTGTTGTC	224-232
	Microsat 1-R	TCCATGCTTCCTACTGTCTG	
JN181247	Microsat 2-R	GAGCGCCAGAGAAGAGGTCC	221-227
	Microsat 2-R	GTTACTTGCTGCGGACTTGC	
JN181243	Microsat 3-R	TCTACAGGCTCAAGGCCATC	215-239
	Microsat 3-R	GAACAAGGGTGCTTGGTCG	
JN181248	Microsat 4-R	AGCACCACGTCGAACCTAC	193-203
	Microsat 4-R	GCGTGCATGCTAGTAGAAAC	
JN181244	Microsat 5-R	GTGTGGTCCTTGCTTCGTC	148-157
	Microsat 5-R	GCATGCTTGACAGCTCTGG	
JN181246	Microsat 6-F	TAACATGCAGCAGGAGGGG	228-260
	Microsat 6-R	ACAGGAACAGCGCGGTATG	
JN181249	Microsat 7-F	CGTGTTTGATCGTGACTGCG	240-250
	Microsat 7-R	TTGGCTCTCTTTCGTCGGG	
JN181250	Microsat 8-F	GCCCAACTATGATTGTGCCG	178-191
	Microsat 8-R	CGAAAGAGGCGGGATTTGG	

II.2.4 Double digestion RADSeq

The protocol used in this study was the double digestion one (a restriction digest with two enzymes simultaneously) as described by Peterson et al. (2012) reported in the Appendix 3. High-molecular-weight genomic DNA was extracted using Plant Dneasy Mini Kit from Qiagen. The genomic DNA digestion was tested by combining different pairs of enzymes on thirty samples from the Gulf of Naples and ten samples from other localities of the Mediterranean Sea. The enzymes used: SbfI, MseI, EcoRI and PstI in the following combination: SbfI/MseI, MseI/PstI and MseI/EcoRI. 48 uniquely barcoded adapter P1 oligo pairs and common adapter P2 were used.

II.2.5 Data analyses

The raw data sequences from psbA and ITS were checked using CHROMAS Lite V2.3 (Technelysium Pty Ltd, Queensland, Australia). If a nucleotide could not be unambiguously determined from the chromatograms, the site was coded with IUPAC ambiguity codes (IUPAC-IUB 1968) and was treated as uncertainty in the analyses. The sequences were aligned in BIOEDIT 7.0.9 (Hall 1999) using the CLUSTAL W Multiple Alignment option with the default settings (Thompson et al. 1994) and then adjusted by eye.

The genetic diversity measures including the numbers of haplotypes (H), the haplotype diversity (Hd), the number of polymorphic sites (S) as well as the nucleotide diversity (π) were calculated using ARLEQUIN 3.5 (Excoffier et al. 2005).

The molecular phylogenetic analysis were inferred by network and cladograms. In order to assess the phylogenetic position of *Cystoseira* species from the Gulf of Naples and to clarify their molecular characterization, psbA sequences were compared with that derived from specimens collected in other localities of the Mediterranean Sea as published by Draisma et al. (2010). GenBank accession number of the sequences used for comparison are listed in the Table II-4.

Phylogenetic analyses were inferred by using Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (HKY85) (Hasegawa et al. 1985). The model of evolution was selected on the basis of Bayesian Information Criterion as implemented in MEGA V. 6 (Tamura et al. 2013). A discrete Gamma distribution was used to model evolutionary rate differences among sites with 5 categories. Bootstrap analysis based on 1000 resampling of the data set was applied (Felsenstein 1981). *Sargassum vulgare* psbA sequence (GenBank Accession Number KJ572518) was chosen as the out-group and *Cystoseira tamariscifolia* psbA sequence (FM958286) was included additionally.

The Bayesian Inference (BI) was performed with MrBayes V. 3.2.6 (Ronquist and Huelsenbeck 2003) using two runs with four Markov Chain Monte Carlo (MCMC) each for 5.000.000 generations under a General Time Reversible Model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites (GTR + I + Γ). The trees were sampled every 1000 generations and finally a consensus tree was generated.

The networks were performed with NETWORK 4.6.1.1 (Forster et al. 2007) using the Median Joining algorithm (Bandelt et al. 1999).

The motif pattern and the repetition of microsatellites were compared to test for differences.

Table II-4. psbA sequences from GenBank used for the comparison of phylogenetic analyses

Species	**GenBank Accession number**
Cystoseira abies-marina	FM958293.1
Cystoseira amentacea	FM958285.1
Cystoseira baccata	FM958291.1
Cystoseira brachycarpa	FM958288.1
Cystoseira compressa	FM958284.1
Cystoseira crinita	FM958287.1
Cystoseira elegans	FM958292.1
Cystoseira humilis	FM958283.1
Cystoseira myrica	FM958278.1
Cystoseira nodicaulis	EU681634.1
Cystoseira tamariscifolia	EU681635.1
Cystoseira zosteroides	FM958290.1
Sargassum vulgare	KJ572518.1

II.3 Results

110 psbA sequences were obtained for 7 species morphologically identified as *Cystoseira amentacea*, *Cystoseira compressa*, *Cystoseira crinita*, *Cystoseira brachycarpa*, *Cystoseira sauvaugeauana* and *Cystoseira spinosa*, further two sequences derived from individuals not unambiguously identified at species level were obtained. The psbA alignment was made up of 930 nucleotides, no gaps were observed. On the basis of morphological identification, the species were divided into three main groups made up by the three most representative species, *Cystoseira compressa*, *Cystoseira amentacea* and *Cystoseira crinita* and the genetic diversity measures for these groups of species are shown separately (Table II-5). Overall psbA showed 49 polymorphic sites and 12 haplotypes that are quite different from each other since the value of haplotype diversity is high (GenBank accession numbers of DNA sequences for psbA from KY657599 to KY657610). *Cystoseira amentacea* had the highest number of haplotypes (5 of which 1 is unique) and 8 polymorphic sites, however the haplotypes slightly differ from each other since the haplotype diversity value is low. *Cystoseira compressa* had two haplotypes different from each other for a single mutation. *Cystoseira crinita* showed 2 haplotypes and 5 polymorphic sites, the average number of nucleotide differences per site or nucleotide diversity (π) is the highest compared to the other two species (Table II-5).

Maximum likelihood psbA phylogenetic tree is coherent with consensus tree build by Bayesian analysis. ML psbA tree clearly separates *Cystoseira* spp. into four distinct clades (Figure II-1). Clade 1 includes species morphologically identified as *Cystoseira amentacea* and the out-group *Cystoseira tamariscifolia.* Clade 2 consists of 10 species identified as *Cystoseira crinita*, two species identified as *Cystoseira brachycarpa*, one individual from Capri referred as *Cystoseira amentacea* and other two individuals not clearly identified at species level. Clade 3 includes the species *Cystoseira sauvaugeauana* and *Cystoseira spinosa* and finally Clade 4 composed by the species *Cystoseira compressa* and the out-group *Sargassum vulgare*. The median joining network identified the same groups as well in terms of haplotypes (Figure II-2). *Cystoseira amentacea* is composed by a main haplogroup including individuals from all the sampling sites and two secondary haplogroups distant for one mutation from the main one including only species coming from Ischia Island. A unique haplotype distant from the main haplogroup for a single mutation includes an individual from the bay of Pozzuoli, in particular from Capo Miseno, Bacoli (BA). *Cystoseira crinita* consisted of a single haplogroup in which there are individuals identified as *Cystoseira brachycarpa* and individuals not clearly identified, two single haplotypes differentiates from

the main haplogroup for three and two nucleotide mutations respectively, in particular the latter one comprises one individual from the locality Bagni Tiberio, Capri, identified as *Cystoseira amentacea*. *Cystoseira compressa* is composed by two main haplogroups distant from each other for a single mutation (a transition T → C) in which individuals from all the sampling sites are included. Finally two single haplotypes corresponding with the species identified as *Cystoseira sauvaugeauana* and *Cystoseira spinosa* are reported. The comparison with data from GenBank showed the same topography of the phylogenetic tree (Figure II-3) and network (Figure II-4) with the differentiation of the species from the present study in 4 clades. Moreover an additional group composed by the species *Cystoseira abies-marina*, *Cystoseira nodicaulis*, *Cystoseira baccata*, *Cystoseira elegans* and the two species of *Cystoseira* from the Gulf of Naples identified as *Cystoseira spinosa* and *Cystoseira sauvaugeauana* collected in the lower infralittoral zone at about 26 and 16 meters depth respectively. Two individuals not clearly identified at species level as well as a single individual previously identified as *Cystoseira amentacea* collapsed in the same group.

Only 30 sequences on a total of 110 has been successfully amplified with ITS regions 1 and 2, alignment required a lot of gaps and analyses were not really clear for this reason were not reported.

On a total of eight microsatellites, seven were successfully amplified on three individuals of *Cystoseira amentacea* from one population of Ischia Island. The motif pattern and repetition is quite different only for one of them (Table II-6).

The double digest RADSeq protocol was entirely applied on the selected samples but the adapters ligation did not work successfully probably because of secondary metabolites contents that inhibit the enzymatic digestion and failed to cut specifically the DNA molecules.

Table II-5. Genetic diversity measures. H: numbers of haplotypes, Hd: haplotype diversity, S: number of polymorphic sites, π: nucleotide diversity.

	H	**S**	**H_d**	**π**
Overall alignment	12	49	0.796	0.016
Cystoseira amentacea	5	8	0.390	0.646
Cystoseira compressa	2	1	0.507	0.507
Cystoseira crinita	2	5	0.222	1.111

Table II-6. Comparison of microsatellites pattern of repeat with the previous study by Robvieux et al. (2012)

	Nucleotide pattern of repeat	
Microsatellite_ID	**Robvieux et al. (2012)**	**This study**
Microsat 1	C_9 A_{10} TGT A_4	C_{13} A_9 TGT A_4
Microsat 2	$(AT)_6$	$(AT)_4$
Microsat 3	$(AG)_{17}$	-----
Microsat 4	G T_5 GTG GCT_5	G T_5 GTG GCT_7
Microsat 5	AGC_7	AGC_6
Microsat 6	$(GA)_{15}$	$(GA)_2$ G_3 $(GA)_5$
Microsat 7	$(CA)_{11}$	$(CA)_{11}$
Microsat 8	$(ACT)_7$	$(ACT)_7$

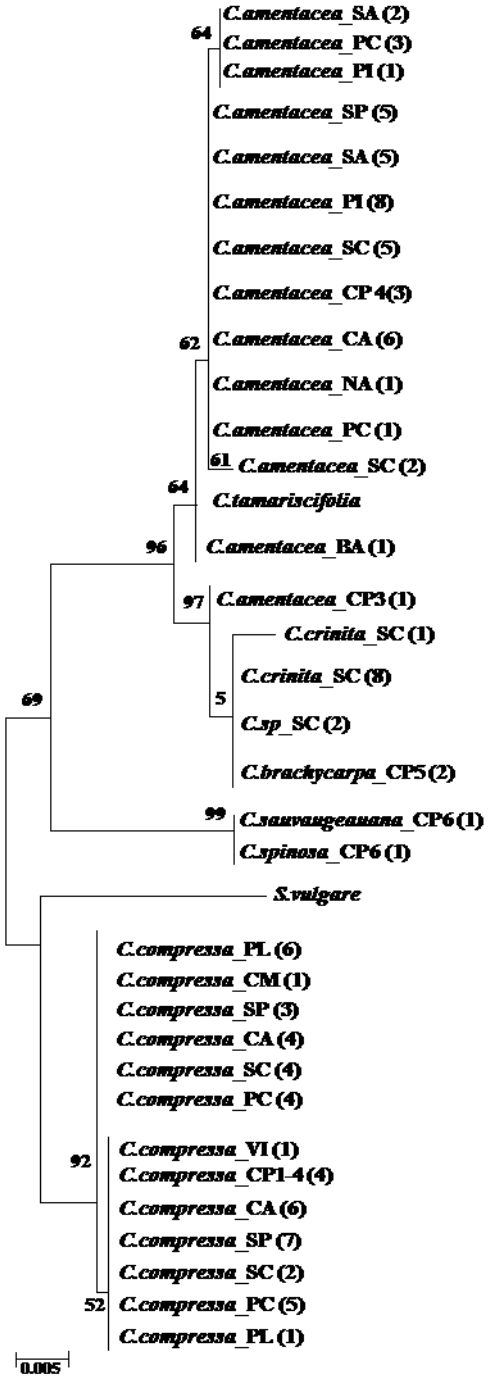


Figure II-1. ML phylogenetic tree based on psbA sequences. The numbers at each node represent the bootstrap value (1000 replicates). The numbers inside brackets represent the number of individuals with identical sequences at the same sampling site.

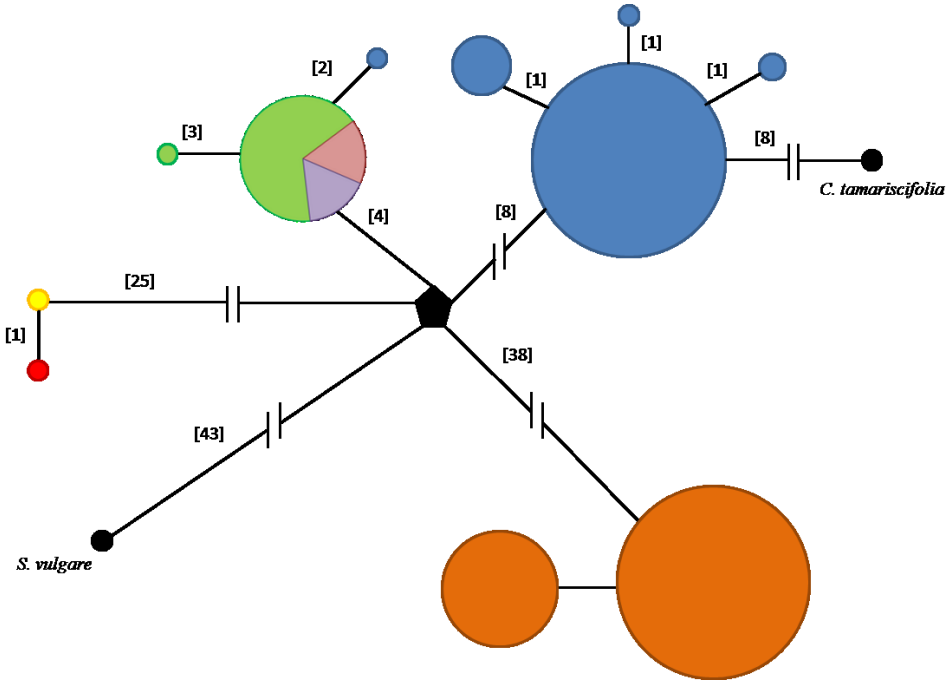


Figure II-2. Median Joining Network. The numbers inside brackets indicate the nucleotide mutations

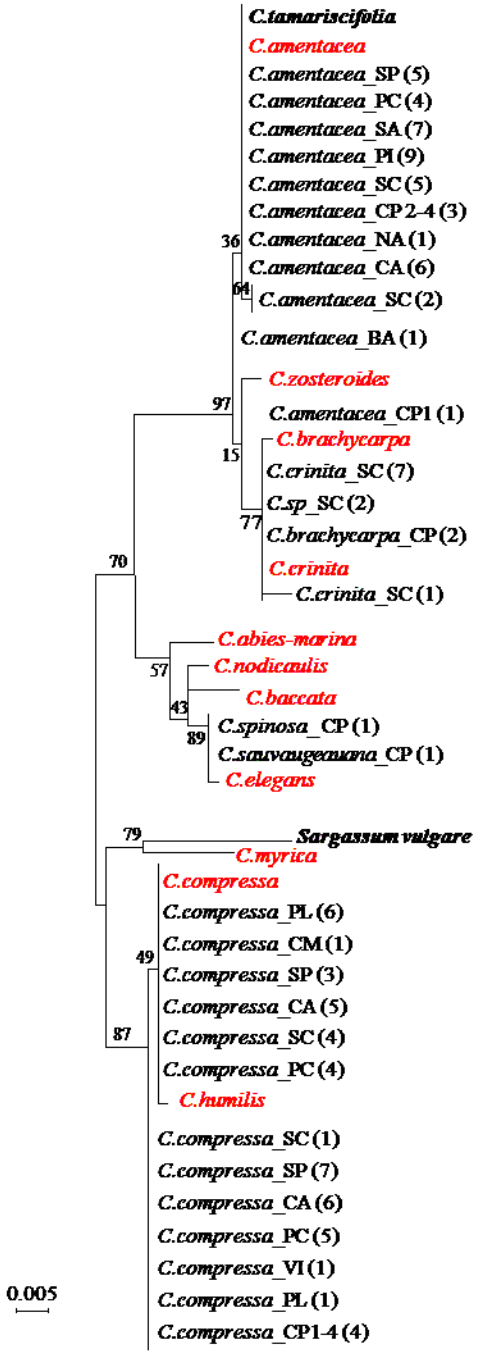


Figure II-3. ML phylogenetic tree based on psbA sequences by comparing data from this study and sequences from GenBank. The number at each node represents bootstrap value (1000 replicates). The number inside brackets represents the number of individuals with identical sequences at the same sampling site. In red the sequences from GenBank, in bold the outgroups.

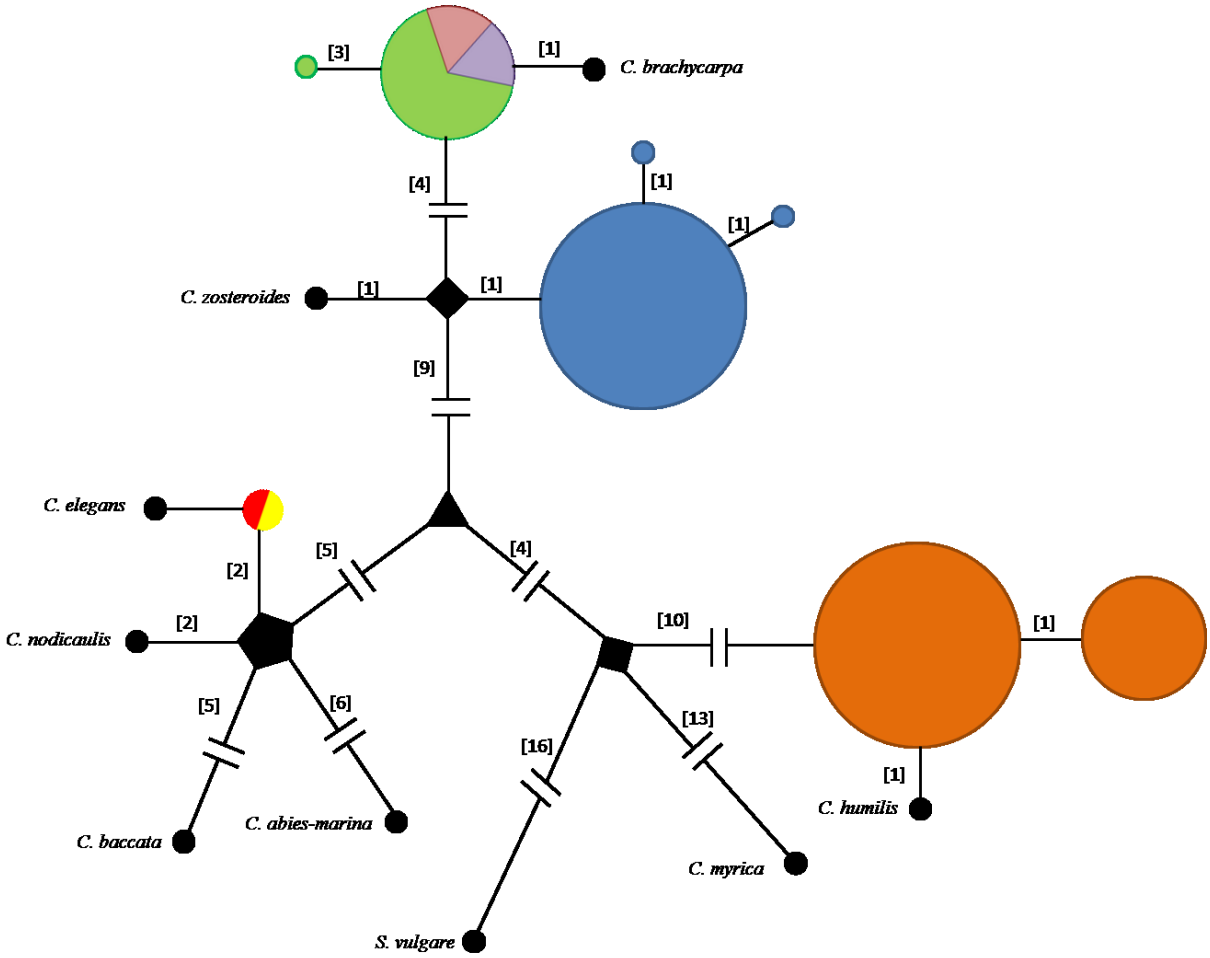
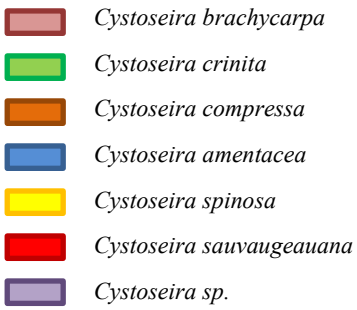


Figure II-4. psbA Median Joining Network by comparing data from the present study and data from GenBank.



II.4 Discussion

Macroalgae of the genus *Cystoseira* are important ecosystem engineering species along the temperate rocky coasts all over the Mediterranean Sea (Giaccone and Bruni 1973b, Ballesteros 1992). Several Mediterranean *Cystoseira* population have declined within the past few decades and one of the most critical threat in determining the disappearance of these species seems to be the natural habitat destruction (Thibaut et al. 2005, Mangialajo et al. 2007, Thibaut et al. 2015). Fragmentation of natural habitat leads to a reduction in population size and increased spatial isolation of populations occupying remaining habitats (Amos and Harwood 1998).

Furthermore, the persistence of any population requires the recruitment of new individuals by means of local reproduction or immigration from other populations (Johnson and Brawley 1998).

The fitness of *Cystoseira* species in terms of reproductive effort and as a consequence recruitment depends both on the position on the vertical gradient and on the distance from natural neighbor populations as also reported for other Fucales (Serrao et al. 1996, Mangialajo et al. 2012). The dispersal of Fucales is considered to be very limited, due to the big size of eggs and zygotes that can rapidly sink (Johnson and Brawley 1998). The consequence of this short-distance dispersal reproductive strategy is the development of monospecific stands close to the parent plants (Pearson and Serrao 2006).

In the sites explored for this study, *Cystoseira compressa* generally builds dense and continuous belts, *Cystoseira amentacea* belts are dense but more often distributed in patches. *Cystoseira compressa* is more tolerant to the stressful conditions as suggested by the previous studies (Thibaut et al. 2005, Pinedo et al. 2007, Mangialajo et al. 2008a) and it is able to colonize even artificial substrates (Susini 2006, Mangialajo et al. 2012). Anthropogenic disturbances can therefore lead to the replacement of *C. amentacea* by *C. compressa* (Mangialajo et al. 2008a).

The direct consequence of this species shift is the decrease of genetic variability and an increased inter-population genetic divergence (Young et al. 1996). Genetic diversity defined as any measure to quantify the genetic variability within a population represents a key element of biodiversity and a fundamental source to explore it (Hughes et al. 2008).

The present study aims to analyze the genetic variability of engineering macroalgae of the genus *Cystoseira* along the coasts of the Gulf of Naples since they are being lost mainly as a consequence of the destruction of the natural habitat of colonization (Grech et al. 2015).

However the analysis of genetic variability within *Cystoseira* species and overall within the order Fucales is a challenging task because of the polymorphic nature and the phenotypic plasticity of these species that make difficult their correct taxonomical identification.

In a recent paper Draisma et al. (2010) dealt with the complex taxonomy of the genus *Cystoseira*, basing their observations on the molecular data from several specimens collected at various localities of all oceans. The results of their work, are quite impressive. In fact, the 34 species of *Cystoseira* examined splitted into 6 distinct clades, each identifying a distinct genus (Figure II-5). On this basis, authors referred the Indo-Pacific species (clade: *Cystoseira*-1) to the genus *Sirophysalis* Kützing, the Indian species (clade: *Cystoseira*-2) to the genus *Polycladia* Montagne and the Pacific ones (clade: *Cystoseira*-3) to the genus *Stephanocystis* Trevisan. Both Atlantic European and Mediterranean species splitted into other three clades: clade *Cystoseira*-4 (that should be maintain the name *Cystoseira*) and clades *Cystoseira*-5 and –6, for which authors postponed the formal proposal of new genera depending on getting additional anatomical, morphological and reproductive data to better characterize them.

In the present study a molecular genetic approach has been used firstly to characterize the species and secondly to investigate the genetic variability among the fragmented population of *Cystoseira* species in the Gulf of Naples.

The plastidial psbA molecular marker has been used to characterize individuals at species level while ITS, microsatellites and RADSeq have been tested to detect the genetic differences at population level.

The analyses with psbA clearly separate the individuals at species level and are coherent with phylogenetic analyses carried out by Draisma et al. (2010) using the combined analysis of the plastidial psbA gene and the partial mitochondrial m23S (Figure II-5). The molecular characterization of species of the present study is also confirmed by comparing results from the analyses with other molecular markers, in particular the plastidial rbcL and the plastidial spacer Rubisco and the nuclear large subunit (LSU) as described by Susini (2006) (Figure II-6, Figure II-7, Figure II-8).

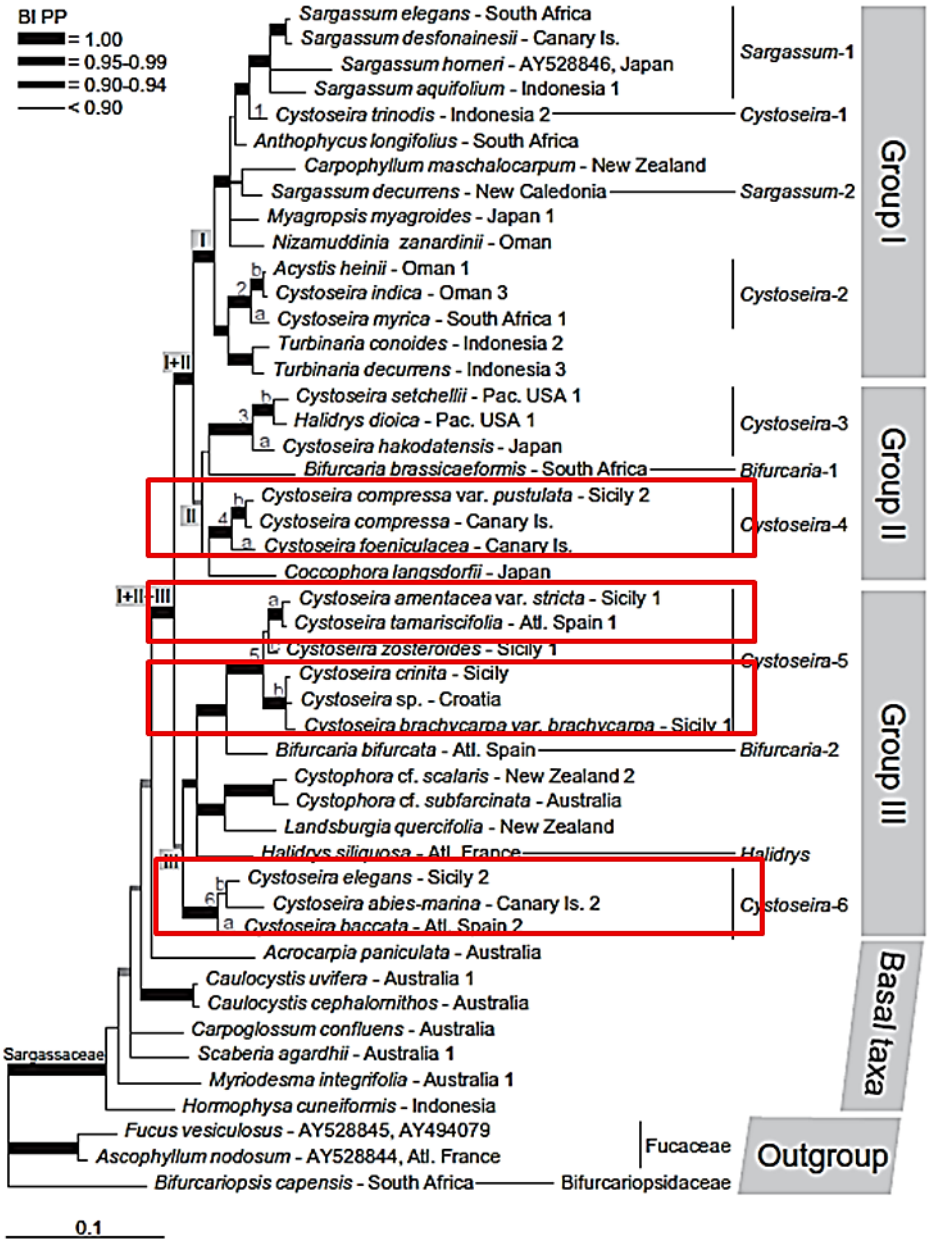


Figure II-5. Bayesian inference majority-rule consensus tree based on plastidial psbA gene and mitochondrial mt23S DNA combined sequence data as described by Draisma et al. (2010), modified.

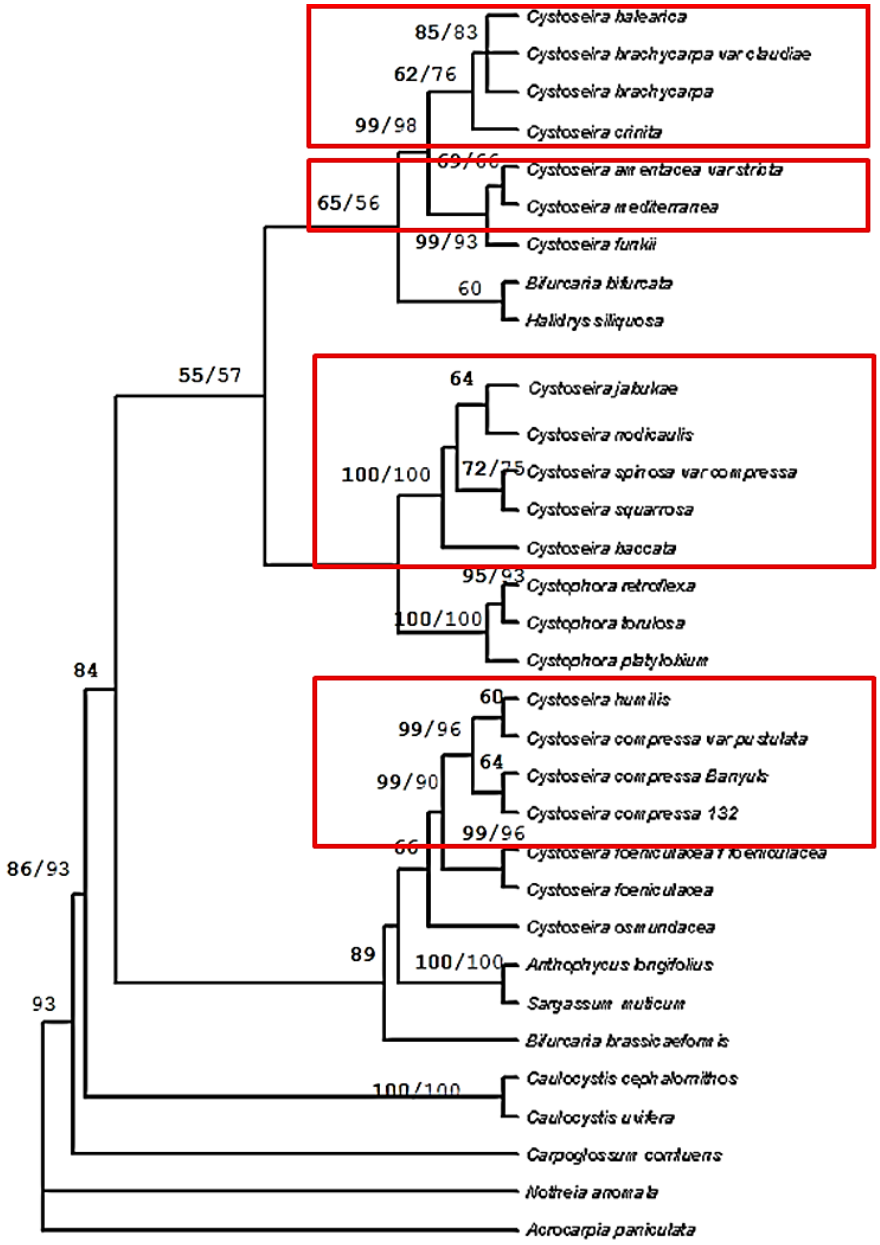


Figure II-6. MP tree obtained for the plastidial rbcL gene as described by Susini (2006), modified.

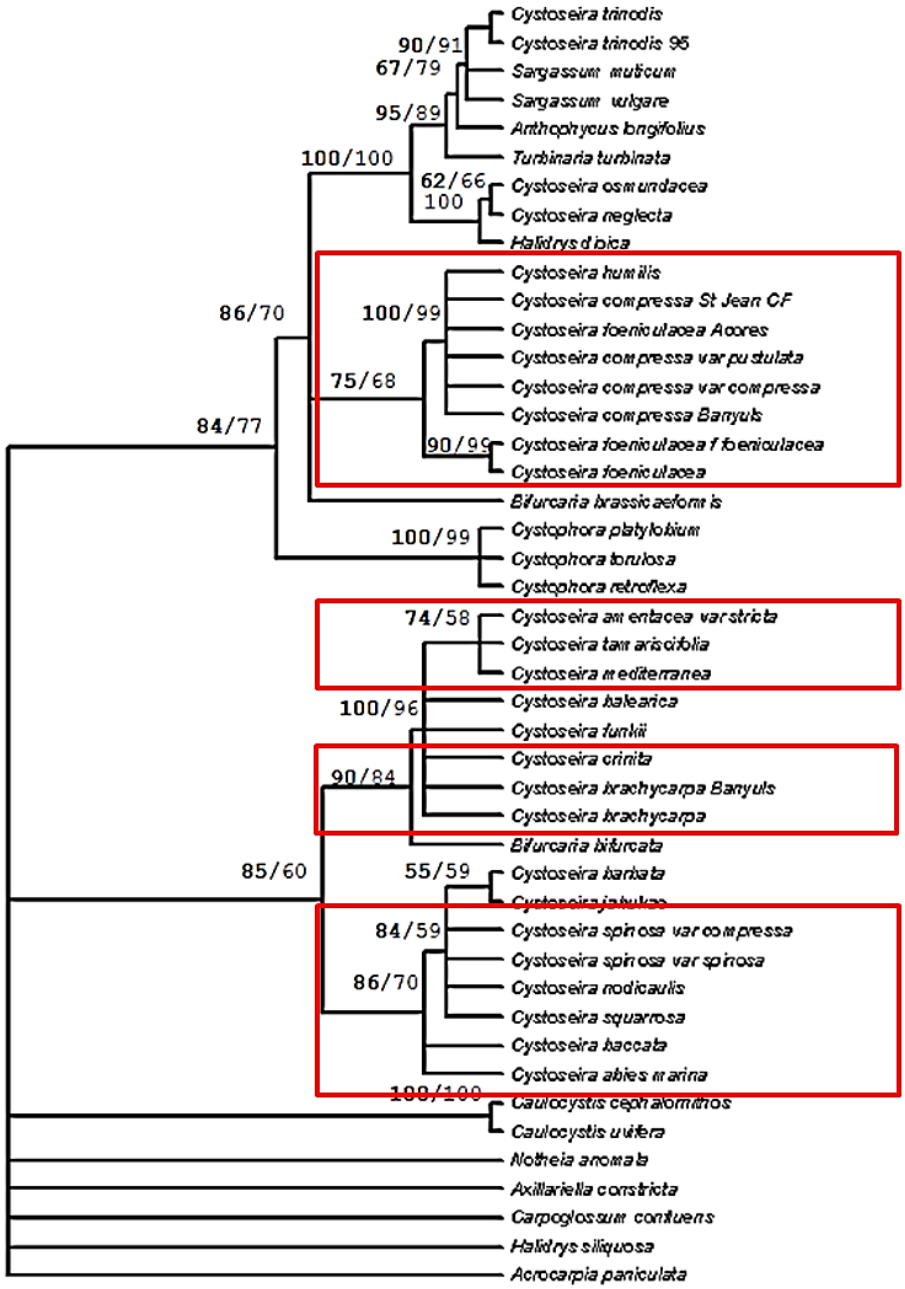


Figure II-7. MP tree obtained for the nuclear LSU as described by Susini (2006), modified.

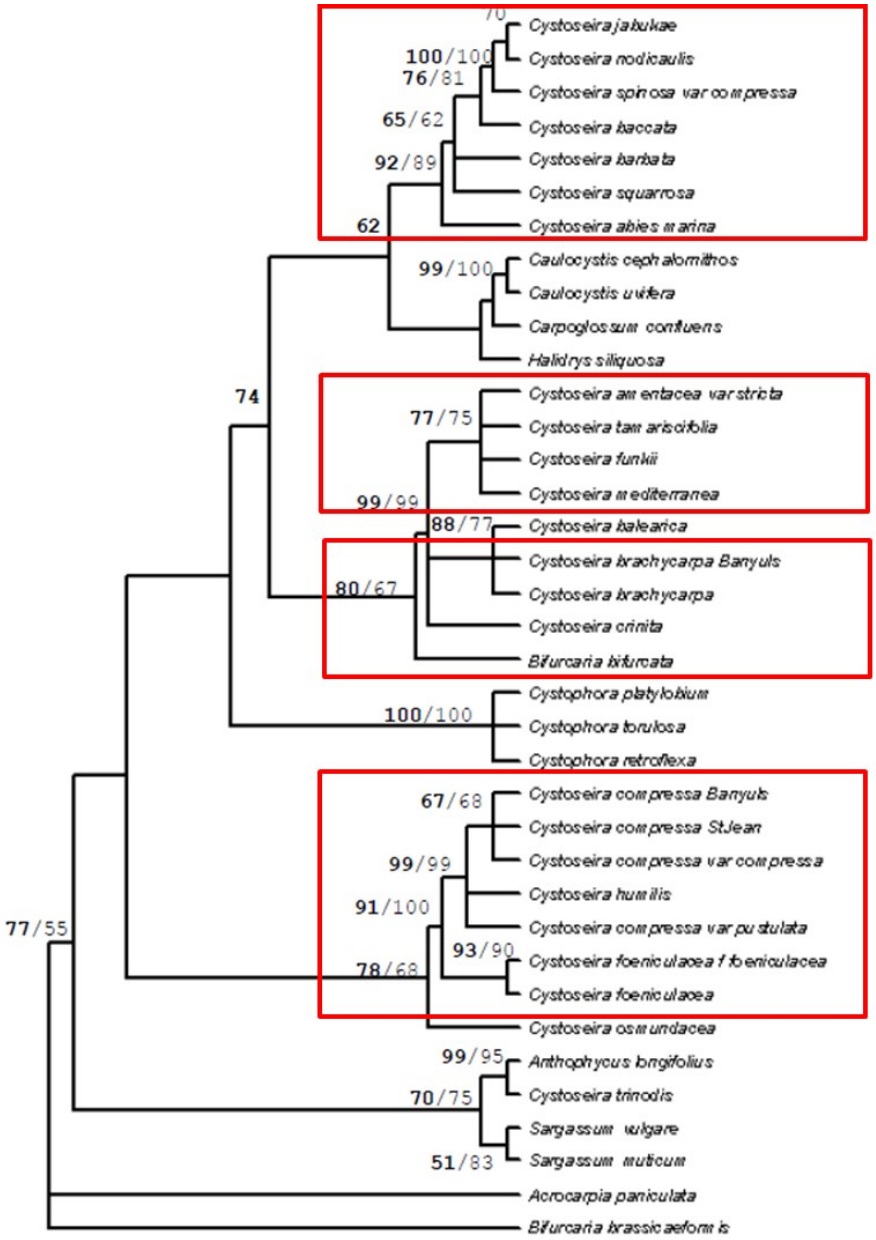


Figure II-8. MP tree obtained for the plastidial spacer of Rubisco as described by Susini (2006), modified.

The comparison with data from literature confirmed the incorrect morphological identification of some species, for example the two specimens identified as *Cystoseira brachycarpa* from Capri Island and two other species from Ischia Island not unambiguously identified at species level, grouped with species identified as *Cystoseira crinita* from the Gulf of Naples, in the same group falls the specimen *Cystoseira crinita* characterized by the studies of Draisma et al. (2010). An individual morphologically identified as *Cystoseira amentacea* from Capri Island clusters within *Cystoseira crinita* clade.

The molecular data of the present study are also validated by the morphological groups described by Giaccone and Bruni (1973b) as well as the chemical data by Amico (1995) (Table II-7).

On the basis of morphological traits, Giaccone and Bruni (1973b) classified the genus *Cystoseira* in four main groups.

In his review, Amico (1995) presents the chemistry of secondary metabolites isolated from the Cystoseiraceae and its contribution to the identification of species of the genus *Cystoseira*. Amico integrates Piattelli (1990) review on the chemistry and the taxonomy of Sicilian *Cystoseira* species.

Also Valls et al. (1993) noticed the close relationship between his chemical classification of the species of the genus *Cystoseira* collected along the French Mediterranean coast and Atlantic coast of Morocco and those based on morphological considerations. To summarize, the morphological and chemical data are closely related and are also related with the phylogenetic data obtained from this study.

PsbA clearly separated the species in four clades as shown in the Figure II-1 and Figure II-3.

The first one comprises the species *Cystoseira amentacea* belonging to the morphologic group I.

The second clade comprises the species *Cystoseira crinita*, two individuals identified as *Cystoseira brachycarpa* and further two individuals not unambiguously characterized from a morphological point of view. These species belong to the morphological group II together with the species *Cystoseira sauvaugeauana*, nevertheless, the individual from the Gulf of Naples morphologically identified as *Cystoseira sauvaugeauana* clusters with the species identified as *Cystoseira spinosa* thus placing in the morphological group III instead of II.

The third clade includes the species identified as *Cystoseira spinosa* and *Cystoseira sauvaugeauana*, for the first one the conformity with the morphological subdivision by Giaccone and Bruni (1973b) is congruent but not for the second one. This lead to suppose an incorrect morphological identification even for this species, however molecular data with psbA cannot be compared since no psbA sequences of species referred as *Cystoseira sauvaugeauan*a is available. A combined molecular analysis with other markers could clarify this ambiguity.

The psbA also gave information on the genetic variability of *Cystoseira* species in the Gulf of Naples stating that *Cystoseira amentacea* and *Cystoseira crinita* are more variable in terms of number of haplotypes and polymorphic sites respect to *Cystoseira compressa* at the same sites. This aspect can be explained through the evolutionary history of these species, *Cystoseira amentacea* together with *Cystoseira mediterranea* are considered to be the

species that had most recently diverged within the genus, on the other side, *Cystoseira compressa* is considered the most ancient species. However the process of speciation within this genus is considered still active today (Ercegović 1959, Roberts 1978, Amico 1995).

Within the genus *Cystoseira*, the chemical data, closely agreeing with the morphological data, proved that species with the most complex metabolites are also the most evolved. Thus *C. amentacea* which elaborates the more complex meroditerpenoids is more evolved than *C. compressa* that does not develops lipophilic secondary metabolites and that is considered to be less evolved.

In the Gulf of Naples recent studies have highlighted a drastic loss of historical occurrence of *Cystoseira* species in the intertidal shore and it corresponds to the highest percentage of coast transformation (Buia et al. 2013b, Grech et al. 2015). Assemblages of *Cystoseira amentacea* and *Cystoseira compressa* still persist in the upper infralittoral shore at some localities along the coasts of Ischia Island. In the rest of the Gulf of Naples isolate patches are present (pers. obs.), for this reason the majority of the samples is referred to the individuals collected along the coast of Ischia Island. Along the coast of Ischia Island, *Cystoseira compressa* is the more widespread species in all the examined sampling sites and it is the less sensitive one to the human modifications. These aspects together with the low genetic diversity, as demonstrated by the psbA analyses, let to suppose that *Cystoseira compressa* represents the most genetically structured species.

Cystoseira amentacea is more distributed in patches, even if these are dense, this species is very sensitive, in fact it is the first one to disappear under human perturbations although this species is the more variable one as detected by the highest number of haplotypes and polymorphic sites.

To conclude, psbA has proven to be a good molecular marker at species level.

Table II-7. Characterization of the genus *Cystoseira* on the basis of morphological features and chemical compounds. Modified by Amico (1995). Chemical groups: Group I = no lipophilic secondary metabolites; Group II = linear diterpenoids; Group III = linear meroditerpenoids; Group IV = tetrahydrofurane, furane and pirane ring; Group V = cyclic meroditerpenoids; Group VI = Bicyclo[3.2.0]heptane ring system; Group VII = Rear- ranged meroditerpenoids. Morphological groups: Group I = *Cystoseira ericaefolia*; Group II = *C. crinito-selaginoides*; Group III = *C. spinifero-opuntioides*; Group IV = *C. discors-abratanifolioides.*

	Chemiotaxonomical group Amico (1995)						
Morphological group	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
Giaccone and Bruni (1973a)							
Group I						***C. amentacea***	*C. mediterranea*
							C. tamariscifolia
Group II		*C. brachycarpa*	***C. crinita***				
			C. sauvaugeauana				
Group III				*C. squarrosa*	***C. spinosa***		
				C. zosteroides	*C. baccata*		
Group IV	***C. compressa***						
	C. humilis						

CHAPTER III

MOLLUSCS COMMUNITY ASSOCIATED WITH THREE *CYSTOSEIRA* ASSOCIATIONS IN THE GULF OF NAPLES (SOUTH TYRRHENIAN SEA)

III.1 Introduction

Marine seaweeds and seagrasses are considered important benthic primary producers along the coasts all over the world (Mann 1973). In the Mediterranean Sea, the species of order Fucales are the dominant ones along the pristine rocky infralittoral shores establishing structurally complex and diversified assemblages and functioning as engineering species (Schiel and Foster 2006). The species of the genus *Cystoseira* together with *Sargassum* are the dominant ones of the order Fucales in the Mediterranean Sea where most of them are endemic (Giaccone and Bruni 1973b) dominating several rocky habitat assemblages from the upper infralittoral shore to the upper circalittoral zone (Verlaque 1987, Ballesteros 1992, Giaccone et al. 1994, Cormaci et al. 2012). They are long-living and very productive macroalgae with a complex tri-dimensional structure providing habitat, food, shelter and nursery for a wide variety of species supporting therefore a high biodiversity (Ballesteros 1992, Bulleri et al. 2002, Mangialajo et al. 2008a, Vergés et al. 2009, Sales et al. 2012). They are considered to have an important ecological role within the European Water Framework Directive (WFD, 2000/60/EC) as coastal water indicator (Orfanidis et al. 2001, Ballesteros et al. 2007, Orfanidis 2007). In the last decades, most of *Cystoseira* assemblages in the Mediterranean Sea are suffering a decline or even worse a real disappearance as an effect of cumulative impact: habitat destruction, eutrophication, water turbidity, overgrazing by sea urchins, outcompetition by mussels, non-indigenous species, human trampling are to be considered as the major threats (Cormaci and Furnari 1999, Thibaut et al. 2005, Airoldi et al. 2008, Mangialajo et al. 2008a, Falace et al. 2010, Giakoumi et al. 2012, Sala et al. 2012a, Buia et al. 2013b, Tsiamis et al. 2013, Bianchi et al. 2014, Grech et al. 2015, Thibaut et al. 2015). These impacts act over time and in unison, with a possible synergistic effect on the species, the ecosystems and their ability to sustain biodiversity. One of the most clear effect is the replacement of canopy forming algae with less structured and opportunistic species such as turf-forming filamentous seaweeds, mussels or sea urchin barrens involving a simplification of the architectural complexity of the communities (Micheli et al. 2005, Perkol-Finkel and Airoldi 2010, Sala et al. 2012a). The loss of habitat structuring species as *Cystoseira* assemblages implies the loss of the associated epibenthic diversity too.

The value of *Cystoseira* associations as a nursery for fish (Orlando-Bonaca and Lipej 2005, Lipej et al. 2009, Riccato et al. 2009, Vergés et al. 2009, Cheminée et al. 2013) as well as the importance in structuring invertebrate communities (Milazzo et al. 2000, Chemello and Milazzo 2002, Fraschetti et al. 2002, Gozler et al. 2010, Urra et al. 2013, Pitacco et al. 2014) has already been investigated in different areas of the Mediterranean Sea.

Amongst the invertebrate fauna inhabiting *Cystoseira* associations molluscs are one of the best represented and dominant taxa, moreover they are considered an important food source for the higher trophic levels.

However there is a gap of knowledge regarding most sites of the Tyrrhenian Sea, among them the Gulf of Naples where invertebrate fauna associated with canopy-forming algae of the genus *Cystoseira* has never been investigated.

The recent study to outline the historical changes in macroalgal diversity in the Gulf of Naples highlighted a drastic decrease of *Cystoseira* species in the infralittoral zones. The decline seems to correspond with the loss of the natural habitat and the consequent coastal transformation (Grech et al. 2015).

The importance of these algal species in structuring complex and diversified habitat and their disappearance in the Gulf of Naples on the other part, make fundamental an investigation of these assemblages as well as of the associated fauna biodiversity.

In order to assess the potential loss of biodiversity associated with these systems in the Gulf of Naples, the aims of the present work are to:

a) Characterize molluscs assemblage structure associated with three *Cystoseira* species along the coasts of Ischia Island in the Gulf of Naples where continuous belt of these algae still persist
b) Determine whether, at a small spatial scale of observation, the three algal species at different sampling sites support a different pattern of associated diversity.

III.2 Materials and Methods

III.2.1 Study sites and sampling design

The study site is Ischia Island, a volcanic island in the south Tyrrhenian Sea. It is located in the northern part of the Gulf of Naples, about 30 kilometres from the city of Naples. It is the largest amongst the Phlegrean Islands. Ischia Island has about 34 km of coastline and a surface area of 46.3 Km^2. In 2007 was established a marine protected area, Regno di Nettuno, including the Island of Ischia and Procida and the islet of Vivara (Figure III-1). The marine protected area Regno di Nettuno is composed by five zones as shown in the Figure III-1. The zone A in which only relief work and surveillance, service activities and scientific research can be performed on behalf of the managing entity under a specific authorization. In the zone B are possible all the activities allowed in the zone A, bathing, underwater guides tours and diving organized by diving centers, sailing and fishing under specific restrictions.

The zone B n.t. is a zone with particular limitation where professional fishing sports practiced by any means, aquaculture and mussel farming, scuba diving with breathing apparatus are forbidden, the latter is exclusively possible with authorized diving centers. The zone C and D are supervised by rules that allow the recreational use in line with the requirements of eco-compatibility.

The morphology of the coasts is heterogeneous and it is strictly subject to the geological history of this island. Generally it is possible to identify four main geographic sectors.

The eastern side is characterized by low rocky coasts and few little sandy beaches. It hosts the biggest harbor of Ischia island that daily connect the island with the mainland. The eastern side of the island falls into the area C of the marine protected area apart from two banks included in the area A.

The morphology of the northern side is similar to that of the eastern part with low rocky coasts and little sandy beaches, this side is characterized by the highest percentage of artificial structure on the coastline, in fact only few scattered individuals of *Cystoseira* species has been detected. The northern coasts fall into the area C of Regno di Nettuno.

The western side is delimited by Punta Caruso and Punta Imperatore. It is characterized by very high rocky coasts and two long sandy beaches. This sector comprises both zones B and C of the marine protected area.

The coast morphology of the southern sector is characterized mainly by high rocky coasts and the biggest sandy beach of the island, the Maronti beach. In this side there is a B n.t zone (the rest of the coasts are under the area B and C.

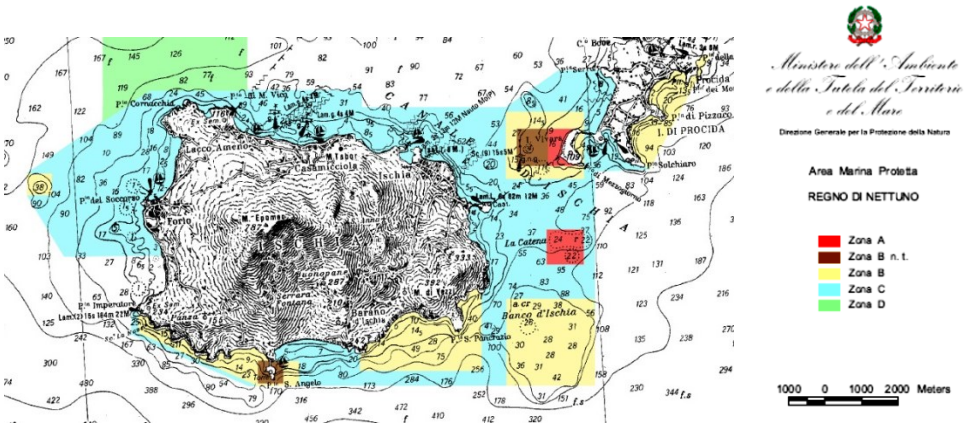


Figure III-1. Regno di Nettuno Marine Protected Area.

Six sampling sites along the coasts of Ischia Island were selected according to the previously known presence and co-existence of the assemblages dominated by the three algal species *Cystoseira amentacea*, *Cystoseira compressa* and *Cystoseira crinita* (Buia et al. 2013b).

Castello Aragonese – CA, San Pancrazio – SP, Sant'Angelo – SA, Scannella – SC, Punta Imperatore – PI, Punta Caruso – PC (Figure III-2).

The sampling covered most of the island coastline excepting the northern side where few scattered or even no individuals were recorded . Sant' Angelo falls into the B n.t. zone of the marine protected area, while Scannella and S. Pancrazio into the zone B, the rest of the sampling sites are located in the zone C.

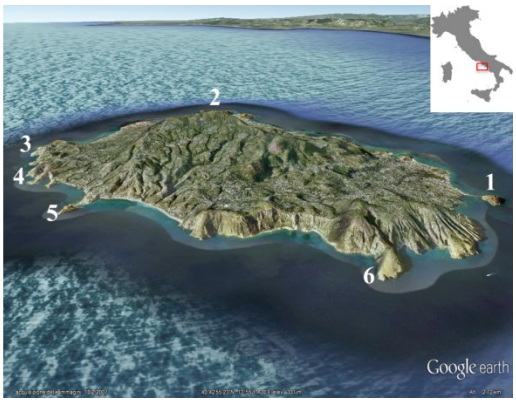


Figure III-2. Ischia Island with the six sampling sites. 1: Castello Aragonese – CA; 2: Punta Caruso – PC; 3: Punta Imperatore – PI, 4: Scannella – SC; 5: Sant'Angelo – SA; 6: San Pancrazio – SP.

To avoid the potential bias related to the seasonal variation, the sampling has been carried out during late spring/beginning summer of 2015 and 2016 since this period corresponds with the maximum developmental stage of *Cystoseira* species (Ballesteros 1992, Hoffmann et al. 1992, Falace et al. 2004, Sales and Ballesteros 2012). Moreover all the chosen sampling sites share some common physical features such as the substrate incline (ranging from 0 to 30 degrees) and the hydrodynamism (mid to high exposed rocky shores). *Cystoseira amentacea* and *Cystoseira compressa* were collected by snorkeling in the upper sublittoral zone (0 meters depth) at the six sampling sites characterized by the co-existence of dense belts of these two algal species. *Cystoseira crinita* was collected by snorkeling only at Scannella (SC) within a tide pool in the proximity of the sea surface since this species was only found at that site. At each site three samples (replicates) were randomly collected by scraping off the macroalgae and associated sessile and vagile fauna within a 20 x 20 cm frame, this area corresponds to the minimum recommended for sampling Mediterranean infralittoral assemblages (Boudouresque and Belsher 1979, Coppejans 1980, Ballesteros 1992, Bianchi et al. 2003a). The use of the airlift sampler was firstly tested but was avoided because of shallow water condition that made difficult the efficiency of this sampling method. The number of thalli of macroalgae in each frame was assessed in *situ*. The samples were sealed in individual plastic bag with seawater and preserved in a cool box up to their

arrival at the laboratory. The thalli were carefully rinsed in seawater to separate the associated fauna and sorted, the material was sieved through a 0.5 mm mesh and finally the material preserved in 70% absolute ethanol for further taxonomic determination. The maximum height of thalli (the length from the base of the holdfast to the distal tip of the frond) and the dry weight after drying at 60°C for 60 hours were assessed at the laboratory. The molluscs were identified at species level with a stereomicroscope according to Cattaneo-Vietti et al. (1990), Giannuzzi-Savelli (2003), Doneddu and Trainito (2005), Cossignani and Ardovini (2011) and counted. The updated taxonomy and nomenclature was cross-checked with the World Register of Marine Species database WoRMS (Appeltans et al. 2012), last accessed: 30 November 2016. The Check List of European Marine Molluscs CLEMAM (Gofas and Le Renard 2013) was followed for the systematic status of the species, last accessed: 30 November 2016.

According to the feeding guilds as described by Solis-Weiss et al. (2004) and Rueda et al. (2009), the following categories were considered: carnivores feeding on other mobile organisms (C); scavengers feeding on the remains of dead organisms (SC); deposit feeders feeding on the organic particles contained in the sediment (D); ectoparasites and specialised carnivores feeding on much larger organisms on which they live during their life cycle (E); filter feeders capturing the particles in the water column with their gills and/or with mucous strings (FF); micrograzers feeding on microalgae, cyanobacteria or detritus attached to algal fronds (MG), macroalgae grazers (AG).

III.2.2 Analysis of macroalgal features

The density calculated as the mean number of thalli per 400 cm^2, the mean height of thalli (cm) and the dry biomass after drying for 60hr at 60°C were calculated. Since none of the macroalgal measures (except the mean height) displayed normal distribution (Shapiro-Wilks test) and homoscedasticity (Bartlett test) neither after data transformation, a non-parametric PERMutational multivariate ANalysis Of VAriance, PERMANOVA (Anderson 2001a, Anderson et al. 2008) applied on the Euclidean distance matrix of raw data was chosen to test differences among macroalgal features at the six sites. PERMANOVA design included two factors: alga (fixed factor, 3 levels) and site (random factor, 6 levels). P-values were obtained by 9999 permutations of raw data under an unrestricted model. Pair-wise comparisons for all the combinations of alga x site were also performed using t tests and 9999 permutations of the raw data. In order to avoid the potential lack of analysis robustness to heterogeneity of data for unbalanced design (Anderson and Walsh 2013), a reduced

analysis only including data from the site SC where the three algal species occurred simultaneously was also performed, in this case the PERMANOVA design included only the factor alga (fixed, three levels). All the multivariate analyses were carried out by the software PRIMER v 6.1.11 with PERMANOVA + V. 1.0.1 add-on package, developed by the Plymouth Marine Laboratory (Clarke and Gorley 2001). The tests for normality and homoscedasticity of data were performed using R V. 3.2.2 (R-Core-Team 2013).

III.2.3 Analysis of the associated assemblages structure and species diversity

Data from the two sampling years were cumulated, as a result at each sites six replicates were considered instead of three. The species were quantified according to: abundance (total number of individuals collected), frequency index (percentage of samples in which a particular species is present) and dominance index (percentage of individuals of a particular species within the sample on the total). The diversity patterns and assemblage structure of malacofauna were described through different diversity measures: number of species (S), the exponential Shannon index (ExpH') and the reciprocal Simpson's index of diversity (1/Simpson) following the suggestion of Jost et al. (2010) to estimate the 'effective number of species'. The cumulative ranked species abundance or *k*-dominance curves were performed to extract overall information on pattern of relative species abundance associated with the three algal species. The dominance curves are based on ranking the species in a sample in decreasing order of their abundance, the ranked abundances are expressed as a percentage of the total abundance of all the species in the sample, in the case of *k*-dominance curve, the cumulative ranked abundance are used (Clarke 1990). The patterns of diversity at different spatial scale were assessed by analyzing alpha diversity (average number of species per sample unit), gamma diversity (the total number of species within a sampling site) and beta diversity (the changes in species composition between sampling sites). Beta diversity was calculated as the multivariate measure based on the average distance between group-centroids defined by a distance matrix determined with the PERMDISP procedure. The PERMDISP is a test used to compare the sample dispersion of different groups based on a distance matrix, when it is applied on a Jaccard distance presence/absence data matrix, it is directly interpretable as a measure of beta diversity among groups (Anderson et al. 2011).

To visualize the spatial pattern of similarity of mollusc assemblages in the three algal species, non-metric multidimensional scaling (nMDS) plot (Kruskal 1964b) was performed on the distance among centroids matrix derived from a Bray-Curtis similarity matrix using the square-root-transformed abundance data.

Furthermore a similarity percentage analysis SIMPER (Clarke and Warwick 1994) was performed to identify the species responsible for the similarity/dissimilarity within and between the three algal species at the different sites.

Multivariate approaches were also used to appraise the composition of mollusc assemblages associated with the three algal species. A nonparametric analysis of variance, PERMANOVA (Anderson 2001a, 2001b, Anderson et al. 2008) applied on a Bray-Curtis similarity matrix using square-root-transformed abundance data in order to down-weight the abundant species was used, the model included two factors: alga (fixed factor, three levels) and sites (random factor, six levels). Pair-wise comparisons for all the combinations of alga x site were also performed using t tests and 9999 permutations of the raw data. PERMANOVA was also performed to test differences in the values of diversity index applied on an Euclidean distance data matrix. All the multivariate analyses were carried out by the software PRIMER v 6.1.11 with PERMANOVA + V 1.0.1 add-on package, developed by the Plymouth Marine Laboratory.

III.3 Results

III.3.1 Description of macroalgal features

Results from the overall analyses are reported since no significant differences were found by comparing data from the reduced analysis. Measures of the macroalgal features are reported in the Table III-1 and shown in the Figure III-3A-C. No significant density differences were found among sites ($F_{5,65} = 0.89$, $p > 0.05$) neither among algae ($F_{2,65} = 4.05$, $p > 0.05$). The average height was significantly different both among algae and sites ($F_{2,65} = 20.07$ $p < 0.01$ and $F_{5,65} = 5.3735$ $p < 0.001$ respectively). Biomass showed significant differences among the six sampling sites ($F_{5,65} = 6.42$, $p < 0.001$) with a maximum resemblance distance between *C. compressa* and *C. amentacea* at PI, however no significant differences were found among algae ($F_{2,65} = 0.0088$, $p > 0.05$), for further details see Table III-2. PERMANOVA results of the pair-wise t-test applied on macroalgal features for the interaction alga x site are reported in the Appendix 4. *Cystoseira compressa* reaches the highest mean values of density in almost all the sampling sites apart from PI and SP where *Cystoseira amentacea* mean values of density are slightly higher. *C. amentacea* reaches the highest mean values of height in all the sampling sites compared to *C. compressa*, however at SC *Cystoseira crinita* has the highest mean value of thalli height. At SP and PI, *C. amentacea* mean value of dry biomass are higher than those of *C. compressa* at the same

sampling sites, however at the other sites *C. compressa* reaches higher values of dry biomass. At SC the mean value of biomass are comparable among the three algal species.

Table III-1. Mean value (± SD) of macroalgal features at the six sampling sites. *Cystoseira compressa: C. co*, *Cystoseira amentacea*: *C. am*, *Cystoseira crinita*: *C. cr*.

	Density (Nr. thalli 400 cm²)			**Height (cm)**			**Biomass (g dw·400 cm^2)**		
Site	*C. co*	*C. am*	*C. cr*	*C. co*	*C. am*	*C. cr*	*C. co*	*C. am*	*C. cr*
CA	9.2 ± 5.1	5.2 ± 4.5	----------	12.4 ± 3.4	13.8 ± 5.4	----------	43.6 ± 13.5	32.0 ± 20.8	----------
SP	5.2 ± 1.5	5.3 ± 2.2	----------	9.9 ± 2.5	14.5 ± 3.7	----------	30.5 ± 7	39.3 ± 29.0	----------
SA	7.8 ± 3.9	6.0 ± 2.4	----------	7.7 ± 2.4	9.3 ± 3.4	----------	19.6 ± 5.8	18.2 ± 6.1	----------
SC	7.0 ± 3.0	4.8 ± 1.6	4.2 ± 1.5	6.2 ± 2.5	9.6 ± 3.0	14.2 ± 3.6	17.7 ± 7.7	19.0 ± 3.7	19.1 ± 6.8
PI	5.5 ± 1.2	5.8 ± 1.9	----------	6.3 ± 1.7	12.0 ± 3.5	----------	18.8 ± 3.0	29.7 ± 13.1	----------
PC	7.5 ± 2.3	4.3 ± 1.2	----------	7.3 ± 1.9	12.3 ± 1.4	----------	20.4 ± 8.7	14.1 ± 3.2	----------

Table III-2. Differences among macroalgal features tested with PERMANOVA for the factors alga (fixed, 3 levels) and site (random, 6 levels) and their interaction (alga x site). Pseudo-F values by 9999 permutation. Df: degrees of freedom. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not-significant.

	Pseudo F-value					
Variables	**Factors**				**Interaction**	
	Alga (df = 2)		Site (df = 5)		Alga x Site (df = 5)	
Density	4.05	ns	0.89	ns	1.13	ns
Height	20.01	**	5.37	***	1.01	ns
Biomass	0.0088	ns	6.42	***	1.48	ns

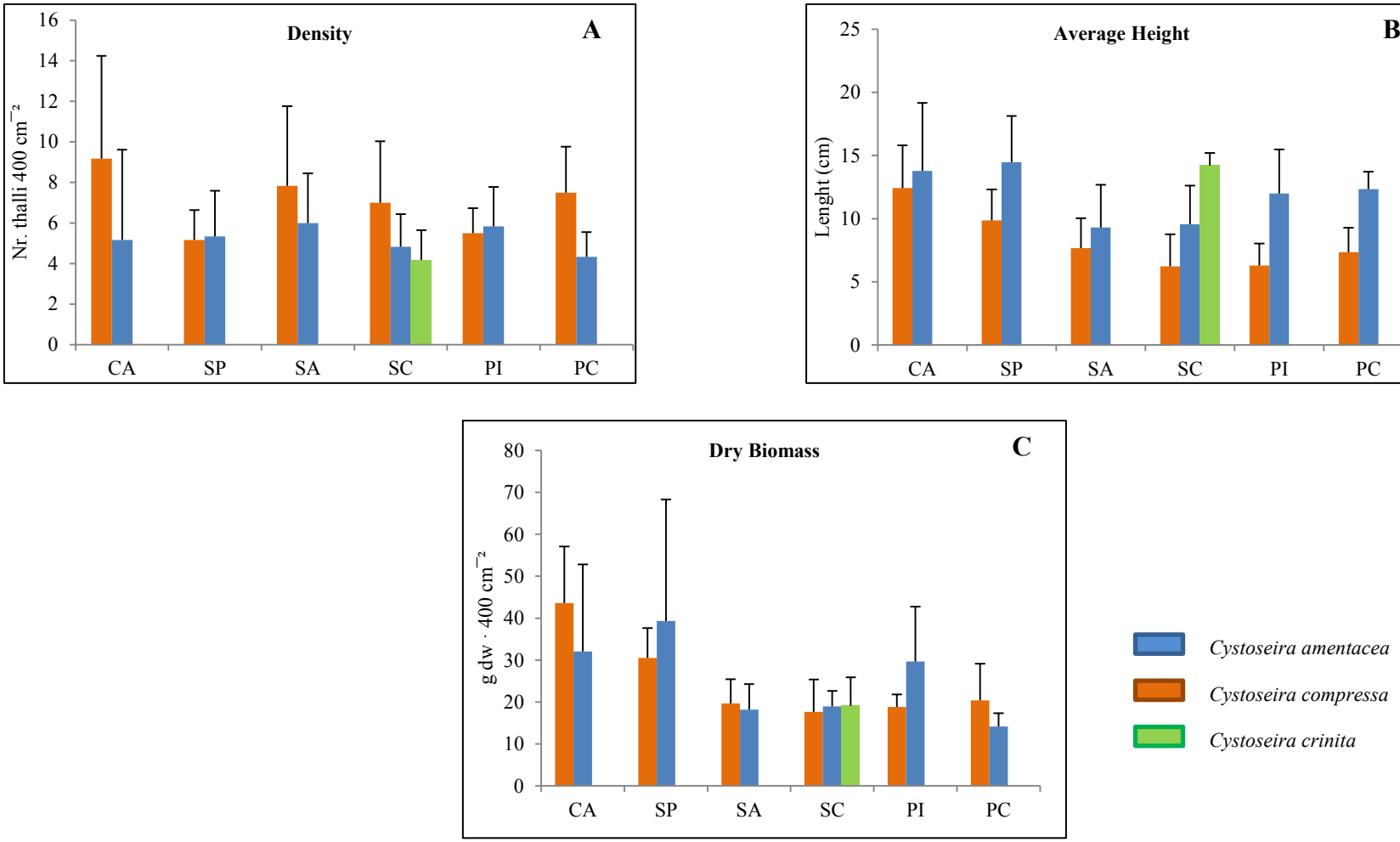


Figure III-3. Mean values ± SD of the three macroalgal measures at the six sampling sites. A: density, B: average height, C: dry biomass

III.3.2 Description of the associated assemblages structure and species diversity

A total of 24837 individuals inhabiting the three associations of *Cystoseira amentacea*, *Cystoseira compressa* and *Cystoseira crinita* along the coasts of Ischia Island were collected. The identified species were 53 belonging to three classes and 31 families: Polyplacophora (2 families), Gastropoda (19 families) and Bivalvia (10 families). Gastropoda was the most species-rich class (38 species), followed by Bivalvia (13 species) and Polyplacophora (2 species). The best represented families were Rissoidae (10 species) and Phasianellidae (3 species) for gastropods and Mytilidae (3 species) for bivalves. A detailed species list is shown in the Table III-3. The Bivalvia was the most important class in terms of abundance with 24104 individuals (97 % of the total abundance), followed by Gastropoda: 729 individuals (2.9%) and Polyplacophora: 4 individuals (0.02%). All the individuals were present at juvenile stage. Most of the identified mollusc species belonged to two main feeding guilds: filter feeders (13 species) and micrograzers (29 species). Only 3 species of carnivores were found, 3 species of scavengers, 3 specialized carnivores (three species of nudibranchs), one species of macroalgae grazers (*Aplysia punctata*) and only one species belongs to deposit feeders (*Scissurella costata*).

The species *Mytilus galloprovincialis* was ubiquitously found within the three algal assemblages at the six sampling sites, moreover it was the most important species in terms of abundance with a total of 23994 individuals contributing to the 96.6% of the total abundance of all the individuals. In order to avoid the potential homogenization of mollusc community biodiversity due to *M. galloprovincialis*, the following analysis did not take into account this species. *Scissurella costata*, *Eatonina fulgida*, *Eatonina pumila* and *Doto rosea* were also ubiquitously found within the three algal species at the six sampling sites.

No significant differences were found in the total number of species associated with the three algae ($F_{2,65} = 3.95$, $p > 0.05$) neither among sites ($F_{5,65} = 0.21$, $p > 0.05$) (Table III-4). Differences were found in the number of individuals with a maximum resemblance distance between *C. compressa* and *C. amentacea* at SP (pair-wise test $t = 2.4$, $p < 0.05$). PERMANOVA results of pair-wise t-tests applied on diversity index for the interaction alga x site for pair of levels of the factor alga are reported in the Appendix 5.

Totally the species associated with *C. compressa* were 33 (22 Gastropoda, 10 Bivalvia and 1 Polyplacophora) representing 24.4% of the total abundances. The same number of species was associated with *C. amentacea* (27 Gastropoda, 5 Bivalvia and 1 Polyplacophora) with a contribution of 67% to the total number of individuals, while 22 was the number of species associated with *C. crinita* (19 Gastropoda, 2 Bivalvia and 1 Polyplacophora) with a contribution of 8.6 % to the total abundances.

Table III-3. List of the species identified within the three algal associations at the six sampling sites in systematical order. Feeding Guild (F. G.): micrograzers (MG); filter feeders (FF); carnivores (C); deposit feeders (D); scavengers (SC), macroalgae grazers (AG); ectoparasites and specialized carnivore (E). Distribution Group (D. G.): shared species among the three algae (A); exclusive species for *C. compressa* (B), exclusive species for *C. amentacea* (C); exclusive species for *C. crinita* (D); shared species *C. compressa*/*C. amentacea* (E), shared species *C. compressa*/*C. crinita* (F); shared species *C. amentacea*/*C. crinita* (G). Sampling sites: Castello Aragonese (CA); San Pancrazio (SP); Sant'Angelo (SA); Scannella (SC); Punta Imperatore (PI), Punta Caruso (PC). + : present; empty: absent;

Family	Species	F. G.	D. G.	CA	SP	SA	SC	PI	PC	%F	%D
Chitonidae	*Chiton olivaceus* (Spengler, 1797)	MG	D				+			1.28	0.12
Acanthochitonidae	*Acanthochitona crinita* (Pennant, 1777)	MG	E	+	+		+			3.85	0.36
Patellidae	*Patella caerulea* (Linnaeus, 1758)	MG	A	+			+		+	3.85	0.36
	Patella sp. (Linnaeus, 1758)	MG	B	+						1.28	0.12
Fissurellidae	*Diodora graeca* (Linnaeus, 1758)	MG	B						+	2.56	0.24
	Fissurella sp. (Bruguière, 1789)	MG	C				+			1.28	0.12
Scissurellidae	*Scissurella costata* (d'Orbigny, 1824)	D	A	+	+	+	+	+	+	56.41	14.35
Trochidae	*Gibbula ardens* (Salis Marschlins, 1793)	MG	A			+	+		+	5.13	0.59
	Phorcus turbinatus (Born, 1778)	MG	F				+	+		3.85	0.36
Phasianellidae	*Tricolia pullus* (Linnaeus, 1758)	MG	C	+		+	+	+		5.13	0.47
	Tricola speciosa (Megerle von Mühlfeld, 1824)	MG	C			+				1.28	0.12
	Tricolia sp. (Risso, 1826)	MG	E		+					2.56	0.24
Cerithiidae	*Bittium latreillii* (Payraudeau, 1826)	MG	D				+			1.28	7.35
	Bittium reticulatum (da Costa, 1778)	MG	E		+		+			2.56	9.37
Cingulopsidae	*Eatonina fulgida* (Adams J., 1797)	MG	A	+	+	+	+	+	+	28.21	0.12
	Eatonina pumila (Monterosato, 1884)	MG	A	+	+	+	+	+	+	38.46	0.12
Rissoidae	*Alvania parvula* (Jeffreys, 1884)	MG	C	+						1.28	0.12
	Alvania sp. Risso, 1826	MG	C			+				1.28	0.12

	Obtusella macilenta (Monterosato, 1880)	MG	G		+	+	+			10.26	2.37
	Obtusella sp. Cossmann, 1921	MG	D				+			1.28	0.12
	Onoba sp. H. Adams & A. Adams, 1852	MG	D				+			1.28	0.12
	Rissoa lia (Monterosato, 1884)	MG	A	+	+	+	+		+	15.38	4.15
	Rissoa variabilis (Megerle von Mühlfeld, 1824)	MG	A	+	+	+	+	+		20.51	3.32
	Rissoa ventricosa (Desmarest, 1814)	MG	E	+		+		+		5.13	0.47
	Setia pulcherrima (Jeffreys, 1848)	MG	A	+	+		+	+		6.41	0.59
	Setia sp. (H. Adams & A. Adams, 1852)	MG	A		+	+	+	+	+	14.10	1.54
Anabathridae	*Pisinna glabrata* (Megerle von Mühlfeld, 1824)	MG	G			+	+			2.56	0.24
Naticidae	*Naticarius hebraeus* (Martyn, 1786)	C	B	+						1.28	0.12
Muricidae	*Stramonita haemastoma* (Linnaeus, 1767)	C	B					+		1.28	0.12
Buccinidae	*Buccinum sp.* (Linnaeus, 1758)	SC	C					+		1.28	0.12
	Euthria cornea (Linnaeus, 1758)	SC	C	+						1.28	0.12
Nassariidae	*Tritia corniculum* (Olivi, 1792)	SC	D				+			1.28	0.12
Columbellidae	*Columbella rustica* (Linnaeus, 1758)	MG	C		+			+		2.56	0.24
Fasciolariidae	*Tarantinaea lignaria* (Linnaeus, 1758)	C	D				+			1.28	0.71
Omalogyridae	*Ammonicera fischeriana* (Monterosato, 1869)	MG	E		+	+		+	+	11.54	1.07
	Omalogyra sp. (Jeffreys, 1859)	MG	A		+	+	+	+	+	14.10	1.66
Aplysiidae	*Aplysia punctata* (Cuvier, 1803)	AG	C						+	1.28	0.12
Dendrodorididae	*Dendrodoris sp.* Ehrenberg, 1831	E	B		+					1.28	0.36
Dotidae	*Doto floridicola* (Simroth, 1888)	E	E	+		+		+	+	7.69	1.78
	Doto rosea (Trinchese, 1881)	E	A	+	+	+	+	+	+	53.85	32.74
Noetiidae	*Striarca lactea* (Linnaeus, 1758)	FF	C		+					1.28	0.12

Mytilidae	*Modiolus sp.* (Lamarck, 1799)	FF	B	+		+			+	7.69	1.78
	Musculus subpictus (Cantraine, 1835)	FF	E	+	+	+	+	+	+	25.64	8.90
	Mytilus galloprovincialis (Lamarck, 1819)	FF	A	+	+	+	+	+	+	100	----
Anomiidae	*Anomia ephippium* (Linnaeus, 1758)	FF	C		+					1.28	0.12
Carditidae	*Cardita calyculata* (Linnaeus, 1758)	FF	G	+			+			2.56	0.59
Cardiidae	*Parvicardium trapezium* Cecalupo & Quadri, 1996	FF	A		+		+			3.58	0.36
Tellinidae	*Macomopsis pellucida* (Spengler, 1798)	FF	B			+				1.28	0.12
Donacidae	*Donax sp.* Linnaeus, 1758	FF	B		+					1.28	0.12
Veneridae	*Dosinia lupinus* (Linnaeus, 1758)	FF	B				+			1.28	0.12
	Callista chione (Linnaeus, 1758)	FF	B			+				1.28	0.12
Corbulidae	*Corbula gibba* (Olivi, 1792)	FF	B					+		1.28	0.12
Thraciidae	*Thracia phaseolina* (Lamarck, 1818)	FF	E		+	+	+			3.85	0.59

The three algal assemblages shared in total 12 species (11 Gastropoda and 1 Bivalvia). The species exclusively associated with one or two algal species were as follow: 11 species exclusively associated with *C. compressa* (5 Gastropoda and 6 Bivalvia), 11 species only associated with *C. amentacea* (9 Gastropoda, 2 Bivalvia), six species only associated with *C. crinita* (1 Polyplacophora, 5 Gastropoda), 8 species shared between *C. compressa/C.amentacea* (1 Polyplacophora, 5 Gastropoda, 2 Bivalvia), the species *Phorcus turbinatus* was only shared among *C. compressa* and *C. crinita*, finally the species *Obtusella macilenta*, *Pisinna glabrata* and *Cardita calyculata* were only shared by *C. amentacea* and *C. crinita*. The most frequent (58.3%) and abundant (30.6%) species associated with *C. compressa* was *Scissurella costata*, followed by *Eatonina pumila* (39% of frequency, 10.7% of abundance) and *Doto rosea* (30.6% of frequency, 9.7% of abundance). *Doto rosea* was the most frequent (80.6%) and abundant (45%) species associated with *C. amentacea*, followed by *Scissurella costata* (50% of frequency, 8.2% of abundance), *Eatonina pumila* (41.7% of frequency, 9.8% of abundance) and *Musculus subpictus* (39% of frequency, 11.4% of abundance). *Scissurella costata* was also the most frequent species associated with *C. crinita* (83.3%), but the most abundant one was *Obtusella macilenta* (17.8%). Values of alpha, beta and gamma diversity as well as the index of diversity are graphed in the Figure III-4.

Table III-4. Differences among diversity index tested with PERMANOVA for the factors alga (fixed, 3 levels) and site (random, 6 levels) and their interaction (alga x site). Pseudo-F values by 9999 permutation. Df: degrees of freedom. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not-significant. Exp H'= exponential Shannon, 1/Simpson= inverse Simpson

	Pseudo F-values					
Variables	**Factors**				**Interaction**	
	Alga (df = 2)		Site (df = 5)		Alga x Site (df = 5)	
Nr. Species	3.96	ns	0.21	ns	1.53	ns
Nr. Individuals	2.085	ns	1.58	ns	2.85	**
Exp. H'	10.88	*	0.66	ns	1.01	ns
1/Simpson	11.49	*	0.73	ns	0.53	ns

Cystoseira crinita at Scannella showed the highest alpha diversity (mean ± SD) of 6 species ± 3, *Cystoseira amentacea* showed the highest alpha diversity at SP (7 ± 4), finally at PC *C. compressa* reached the highest alpha diversity (4 ± 1). The lowest alpha diversity was associated respectively to *C. compressa* at SP with 2 species ± 1 and *C. amentacea* at PC (3

± 1). The reciprocal Simpson's as well as the Exponential Shannon showed significant differences between algae but not between sites (Table III-4). The data suggested highest diversity in terms of 'effective number of species' at SC for *C. crinita* (ExpH' = 6 ± 3, 1/Simpson = 5 ± 3), the lowest diversity was found at SP for *C. compressa* (ExpH' = 2 ± , 1/Simpson = 2 ±1). Beta diversity did not show a significant difference among algae and sites (PERMDISP $F_{12,65} = 1.5$, $p > 0.05$). Gamma diversity or species richness within a site ranged from 23 species at SC associated with *C. crinita*, to 18 species at SP associated with *C. amentacea* and 14 species at SA and PI associated with *C. compressa,* the lowest gamma diversity corresponded to *C. compressa* at SC with 10 species Figure III-4 A-F).

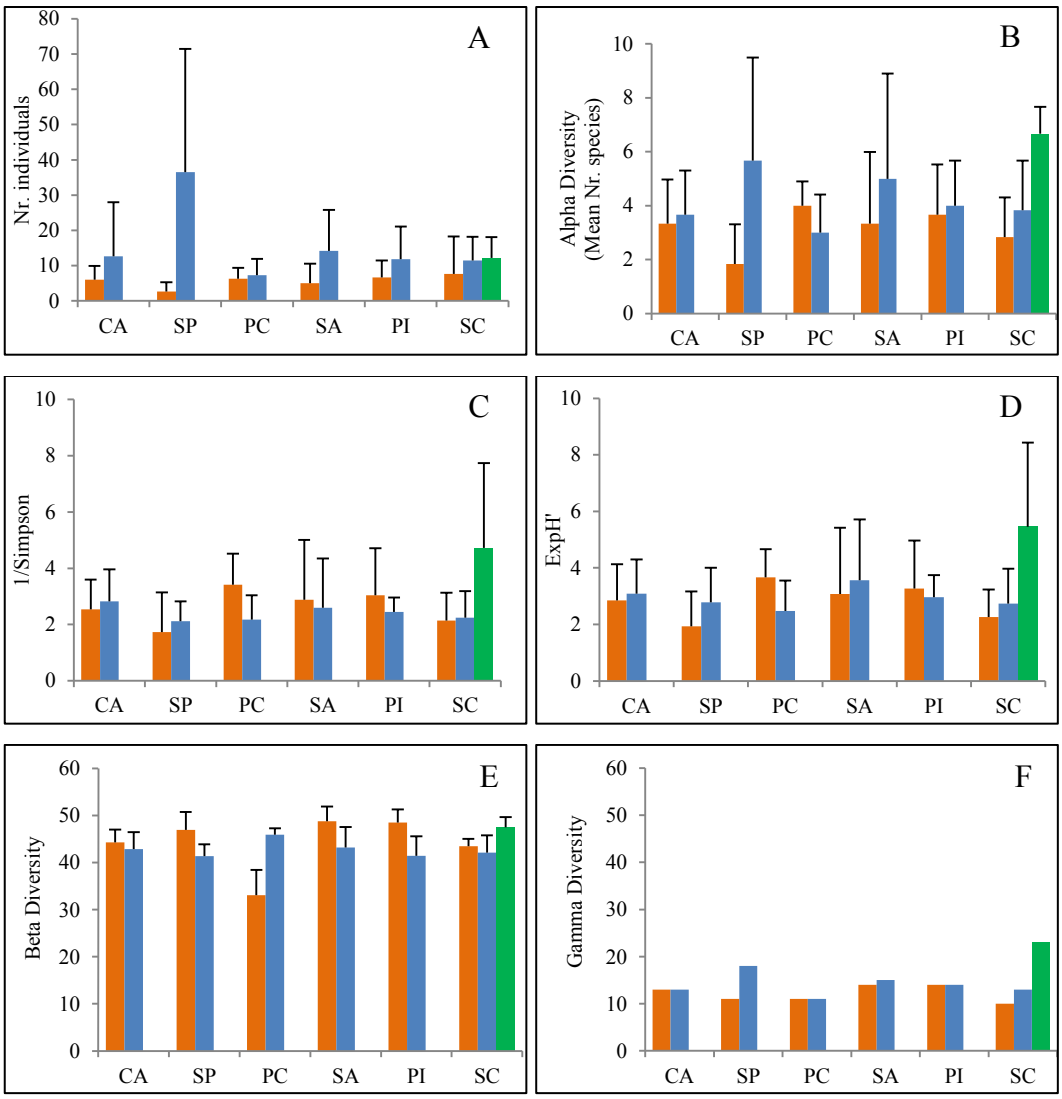
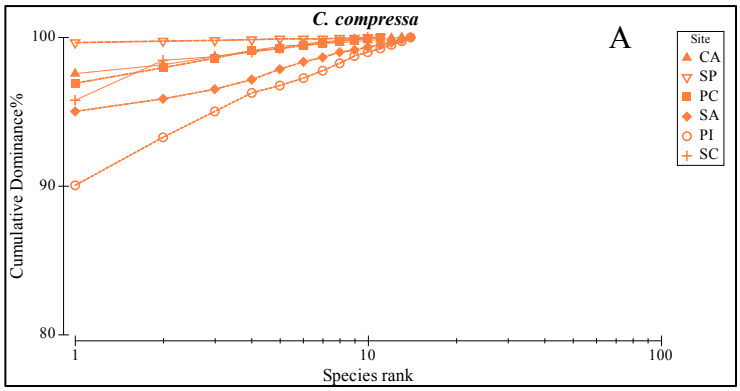
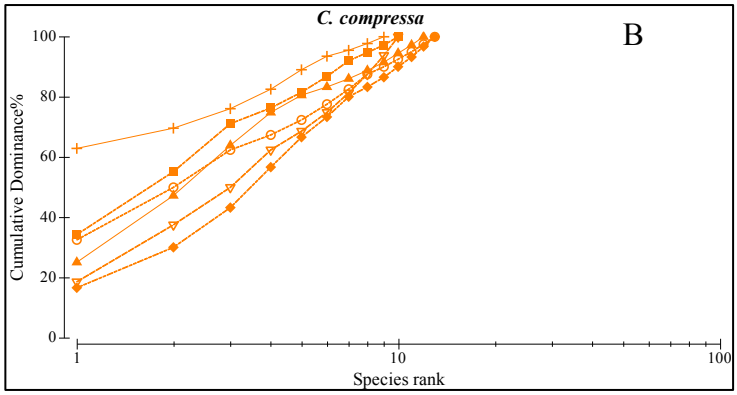
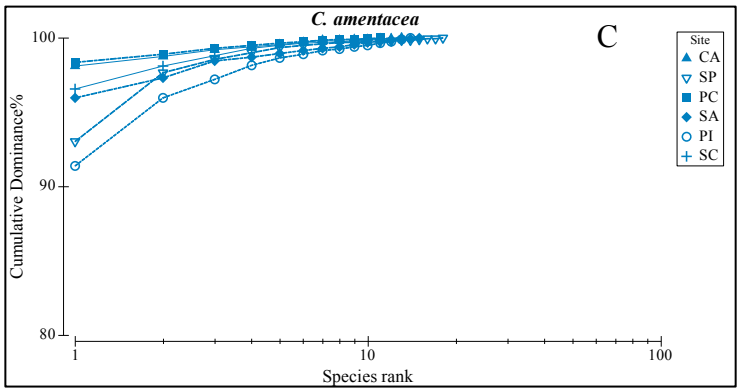


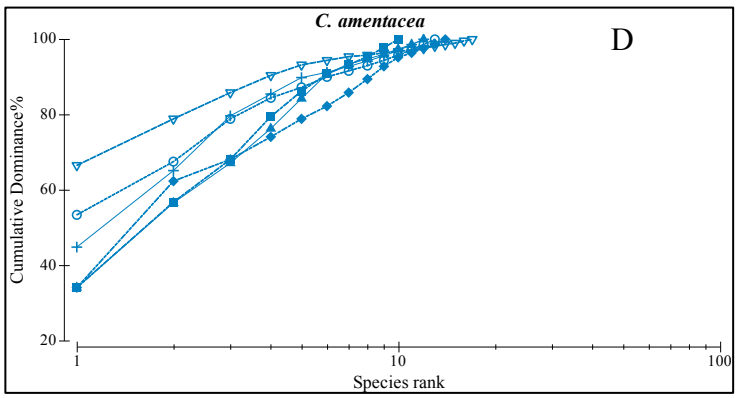
Figure III-4. Mean values ± SD of diversity index. A: number of individuals, B: alpha diversity (mean number of species); C: reciprocal Simpson; D: Exponential Shannon; E: beta diversity (% of unshared species); F: gamma diversity (total number of species).

Two different types of k-dominance curves were plotted, considering or not *M. galloprovincialis* Figure III-5.









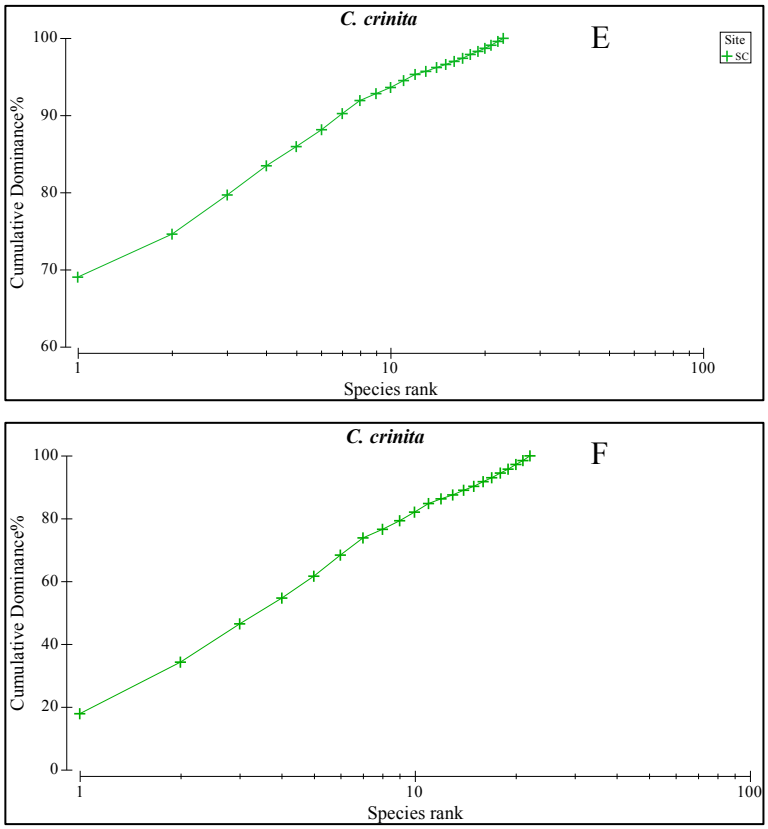


Figure III-5. k-dominance curves considering (A, C, E) or not (B, D, F) *M. galloprovincialis.*

It was clear that the species *M. galloprovincialis* dominated over the others in the three algal assemblages at the six sites with high initial value of dominance and k-dominance curves reaching quickly the asymptote.

Unless the presence of *M. galloprovincialis*, in general the three algae hosted diversified molluscs assemblages in the six sites with low initial dominance and k-dominance curves reaching slowly the asymptote, except for *C. compressa* at SC and *Cystoseira amentacea* at SP where the species *Scissurella costata* (63% of total abundance) and *Doto rosea* (67% of total abundance) were the dominant ones respectively.

Overall the mollusc community structure differed significantly both among algae and sites (Figure III-6). There were significant differences both among algae ($F_{2,65} = 7.27$, $p < 0.01$) and sites ($F_{5,65} = 3.28$, $p < 0.001$) with a maximum distance between *C. compressa* / *C. crinita*.

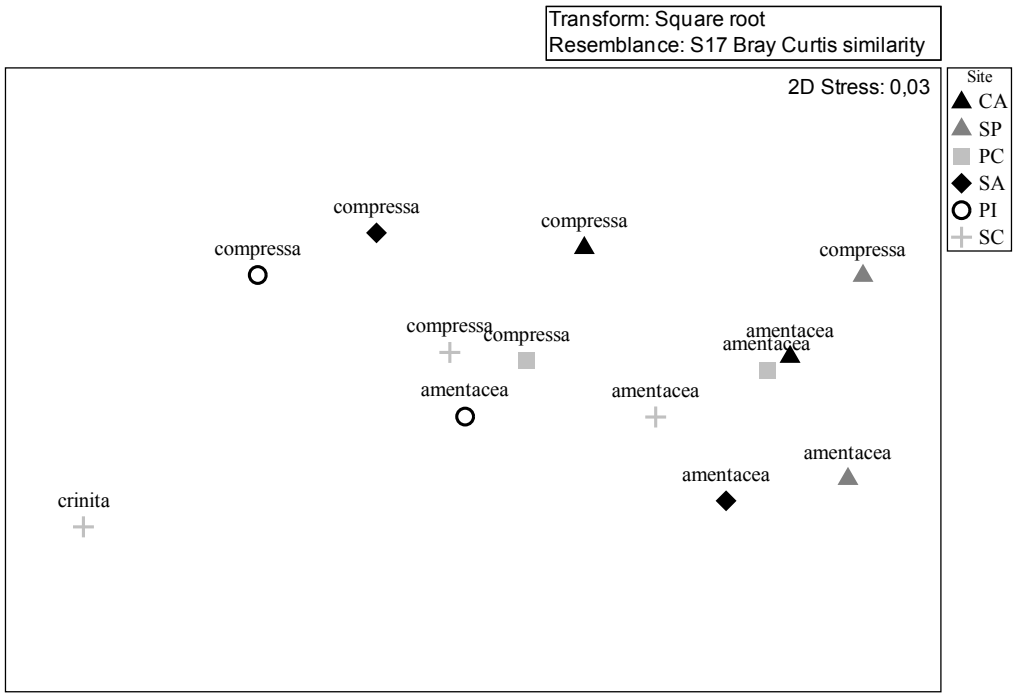


Figure III-6. Non-metric multidimensional scaling (nMDS) plot showing the spatial pattern of similarity of mollusc assemblages associated with the three algal species at the six sites performed on the distance among centroids matrix derived from a Bray-Curtis similarity matrix using square-root-transformed abundance data.

The SIMPER analyses highlighted that *C. amentacea* and *C. crinita* displayed an higher average similarity in species composition (28% and 27% respectively) respect to *C. compressa* (17%). The number of species contributing to the 90% of the similarity between the three algal assemblages are 6 for *C. compressa* and *C. crinita* and 5 for *C. amentacea.* *Scissurella costata* was the most important species in term of percentage of similarity within *C. compressa* and *C. crinita* (46% and 38% respectively), *Doto rosea* contribute with the 54% of similarity for what concern *C. amentacea*. The highest dissimilarity was found among *C. compressa* and *C. crinita* (Table III-5*)*.

Table III-5. Similarity percentage (SIMPER) results

Group *C. compressa*
Average similarity: 17,09 %

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Scissurella costata	0,87	7,86	0,63	45,97	45,97
Eatonina pumila	0,47	3,67	0,37	21,49	67,46
Doto rosea	0,38	2,20	0,30	12,89	80,35
Eatonina fulgida	0,23	0,66	0,18	3,87	84,22
Ammonicera fischeriana	0,17	0,62	0,14	3,63	87,85
Musculus subpictus	0,22	0,56	0,15	3,26	91,11

Group *C. amentacea*
Average similarity: 27,71 %

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Doto rosea	1,98	15,00	1,05	54,13	54,13
Scissurella costata	0,72	3,95	0,49	14,24	68,37
Eatonina pumila	0,71	3,01	0,39	10,87	79,24
Musculus subpictus	0,79	2,34	0,37	8,45	87,70
Eatonina fulgida	0,57	1,91	0,33	6,88	94,57

Group *C. crinita*
Average similarity: 26,94 %

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Scissurella costata	1,21	10,14	1,20	37,66	37,66
Rissoa variabilis	0,67	4,56	0,76	16,91	54,57
Obtusella macilenta	1,09	4,30	0,76	15,94	70,51

Rissoa lia	0,74	3,86	0,78	14,33	84,84
Setia sp.	0,33	1,04	0,26	3,86	88,70
Omalogyra sp.	0,50	0,93	0,26	3,43	92,13

Group *C. compressa / C. amentacea*
Average dissimilarity = 42,95 %

	Group *C. compressa*	Group *C. amentacea*				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Doto rosea	0,38	1,98	16,77	1,26	20,60	20,60
Scissurella costata	0,87	0,72	10,05	0,89	12,34	32,94
Eatonina pumila	0,47	0,71	8,89	0,79	10,92	43,86
Musculus subpictus	0,22	0,79	7,06	0,76	8,67	52,53
Eatonina fulgida	0,23	0,57	6,08	0,73	7,46	60,00
Rissoa variabilis	0,18	0,26	3,35	0,54	4,12	64,11
Ammonicera fischeriana	0,17	0,08	2,93	0,38	3,60	67,71
Rissoa lia	0,11	0,27	2,76	0,43	3,39	71,10
Modiolus sp.	0,26	0,00	2,38	0,39	2,92	74,02
Setia sp.	0,15	0,12	2,13	0,48	2,62	76,64
Doto floridicola	0,03	0,22	1,97	0,39	2,42	79,06
Omalogyra sp.	0,08	0,17	1,95	0,47	2,39	81,45
Setia pulcherrima	0,06	0,06	1,53	0,27	1,87	83,32
Rissoa ventricosa	0,06	0,06	1,04	0,31	1,27	84,59
Tricolia pullus	0,00	0,11	0,96	0,32	1,18	85,77
Thracia phaseolina	0,08	0,03	0,95	0,26	1,16	86,94
Obtusella macilenta	0,00	0,14	0,70	0,31	0,86	87,80
Acanthochitona crinita	0,03	0,06	0,67	0,27	0,82	88,62
Alvania parvula	0,00	0,03	0,57	0,15	0,70	89,32
Patella caerulea	0,03	0,03	0,55	0,22	0,67	89,99
Diodora graeca	0,06	0,00	0,54	0,23	0,66	90,65

Group *C. compressa / C. crinita*
Average dissimilarity = 83,42

Species	Group *C. compressa* Av.Abund	Group *C. crinita* Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Scissurella costata	0,87	1,21	9,69	0,93	11,61	11,61
Obtusella macilenta	0,00	1,09	7,89	1,05	9,46	21,07
Rissoa variabilis	0,18	0,67	6,01	1,15	7,20	28,27
Omalogyra sp.	0,08	0,50	6,00	0,56	7,20	35,47
Rissoa lia	0,11	0,74	5,34	1,16	6,40	41,87
Eatonina fulgida	0,23	0,68	5,33	0,82	6,39	48,26
Eatonina pumila	0,47	0,24	4,68	0,81	5,61	53,88
Doto rosea	0,38	0,33	4,23	0,78	5,07	58,95
Setia sp.	0,15	0,33	4,02	0,69	4,82	63,77
Gibbula ardens	0,03	0,33	2,58	0,70	3,09	66,86
Tarantinaea lignaria	0,00	0,41	2,49	0,44	2,98	69,84
Cardita calyculata	0,06	0,17	2,46	0,46	2,94	72,78
Modiolus sp.	0,26	0,00	1,94	0,41	2,32	75,11
Musculus subpictus	0,22	0,00	1,73	0,42	2,07	77,17
Phorcus turbinatus	0,06	0,17	1,55	0,49	1,85	79,03
Parvicardium trapezium	0,03	0,17	1,52	0,46	1,83	80,85
Setia pulcherrima	0,06	0,17	1,50	0,45	1,80	82,65
Patella caerulea	0,03	0,17	1,48	0,46	1,77	84,43
Ammonicera fischeriana	0,17	0,00	1,47	0,41	1,76	86,18
Chiton olivaceus	0,00	0,17	1,27	0,44	1,52	87,70
Obtusella sp.	0,00	0,17	1,20	0,44	1,43	89,14
Onoba sp.	0,00	0,17	1,20	0,44	1,43	90,57

Group *C. amentacea / C. crinita*
Average dissimilarity = 82,92 %

Species	Group *C. amentacea* Av.Abund	Group *C. crinita* Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Doto rosea	1,98	0,33	11,41	1,25	13,76	13,76
Scissurella costata	0,72	1,21	8,21	0,94	9,90	23,65
Obtusella macilenta	0,14	1,09	6,80	1,01	8,20	31,86
Eatonina fulgida	0,57	0,68	5,80	0,93	7,00	38,85
Eatonina pumila	0,71	0,24	5,25	0,76	6,33	45,19
Rissoa lia	0,27	0,74	5,22	1,25	6,30	51,49
Omalogyra sp.	0,17	0,50	5,02	0,57	6,05	57,54
Rissoa variabilis	0,26	0,67	4,71	1,05	5,68	63,22
Musculus subpictus	0,79	0,00	4,37	0,71	5,28	68,49
Setia sp.	0,12	0,33	3,22	0,66	3,89	72,38
Gibbula ardens	0,04	0,33	2,23	0,70	2,69	75,07
Tarantinaea lignaria	0,00	0,41	2,19	0,43	2,65	77,71
Cardita calyculata	0,00	0,17	1,75	0,41	2,11	79,82
Patella caerulea	0,03	0,17	1,32	0,45	1,59	81,41
Doto floridicola	0,22	0,00	1,29	0,36	1,55	82,97
Setia pulcherrima	0,06	0,17	1,25	0,47	1,51	84,47
Parvicardium trapezium	0,03	0,17	1,25	0,45	1,50	85,97
Pisinna glabrata	0,03	0,17	1,12	0,46	1,36	87,33
Chiton olivaceus	0,00	0,17	1,09	0,43	1,32	88,65
Phorcus turbinatus	0,00	0,17	1,09	0,43	1,32	89,96
Obtusella sp.	0,00	0,17	1,04	0,43	1,25	91,22

III.4 Discussion

Macrophytes are important primary producers along the coasts worldwide serving as habitat or functioning as ecological engineering species. The beds of seagrasses, kelp and fucoids support epiphytic algae and animals, as well as a variety of associated vagile fauna (Christie et al. 2009).

The macroalgae of the genus *Cystoseira* are important engineering species along the temperate rocky coasts all over the Mediterranean sea. Their architectural complex tridimensional structure serve as habitat for a wide variety of organisms both vertebrates and invertebrates (Chemello and Milazzo 2002, Gozler et al. 2010, Cheminée et al. 2013, Urra et al. 2013, Pitacco et al. 2014).

The disappearance of *Cystoseira* species is a phenomenon that has been described in different areas of the Mediterranean Sea, also in the Gulf of Naples (Thibaut et al. 2005, Mangialajo et al. 2008a, Buia et al. 2013b, Grech et al. 2015, Thibaut et al. 2015). The decline of these habitat forming species also imply the loss of the associated faunal biodiversity.

The present study aims to fill the gap of knowledge concerning the invertebrate fauna composition and diversity associated with the engineering macroalgae of the genus *Cystoseira* along the coasts of Ischia Island where belts of these algae are still present and since previously have never been assessed. On the other hand, studies regarding the diversity of invertebrate fauna associated with the seagrass meadow *Posidonia oceanica* at Ischia Island have been the aim of several studies (Mazzella et al. 1989, Gambi et al. 1992, Mazzella et al. 1992, Scipione et al. 1996, Gambi 2002, Vasapollo 2009, Garrard 2013).

The molluscs community associated with the three *Cystoseira* assemblages, *Cystoseira compressa*, *Cystoseira amentacea* and *Cystoseira crinita* along the coasts of Ischia Island has been characterized and the pattern of diversity at a small geographic spatial scale has been analyzed.

It is known that different macroalgae do not support benthic fauna in the same way (Williams and Seed 1992) and this may depend on several factors such as the life cycles, the algal architecture or the exhibition of chemical defenses (Duffy and Hay 1994). Different algal shapes are important in determining patterns of abundance and size structure of the associated fauna (Edgar 1983).

The overall pattern of spatial distribution of these three algal species and their main architectural attributes are quite different within the sampling sites taking into account for the present study. *Cystoseira compressa* was the more widespread species at the six

sampling site holding dense and continuous belts, *Cystoseira amentacea* assemblages were dense but more scattered over the sites, *Cystoseira crinita* only occurred in a tide pool close to the sea surface at Scannella where it totally covers the rocky pool walls. Although *Cystoseira compressa* reached the higher mean values of density in all the sites compared to *Cystoseira amentacea*, it is characterized by shorter and less branched thalli. The biomass does not show significant differences among the three algal assemblages. The height represents thus the most important macroalgal feature in diversifying the algal associations among the six sampling sites.

Although the number of mollusc species associated with *Cystoseira amentacea* and *Cystoseira compressa* was the same, *C. amentacea* host an higher number of individuals at the six sampling sites, probably because the longer thalli of the latter algal species offer a wider surface of colonization. The maximum total number of species (gamma diversity) at local scale as well as the maximum mean number of species per sampling unit (alpha diversity) was found at Scannella for the species *C. crinita*, this data was confirmed by the highest values of the diversity index too. The number of individuals associated with *C. crinita* at SC was comparable to that of the other two algal species at the same site, this data together with the high value of beta diversity (percentage of unshared species within the sampling site) seem to highlight that *C. crinita* malacofauna was more heterogeneous in terms of species composition (an higher number of different species most of which are unshared among the different sampling units). At Scannella the species *C. crinita* has longer thalli than those of *C. compressa* and *C. amentacea*, this could be related to the peculiar habitat of the rocky pool representing a sheltered zone with low hydrodynamic regime and a low competition for space with the other algal species. Apart from *C. crinita* at SC, the highest value of alpha diversity are due to *C. amentacea* at all the sampling sites although the beta diversity is lower in *C. amentacea* respect to *C. compressa*. This data seems to suggest that *C. amentacea* malacofauna is more homogeneous in terms of species composition among the different sites.

The three *Cystoseira* species stands along the coasts of Ischia Island harbor a species-rich malacofauna assemblages, a total of 53 mollusc species were identified. Gastropoda represents the dominant taxa in terms of number of species followed by Bivalvia and Polyplacophora. This trend is confirmed by other studies on the molluscs assemblage associated with photophilous algal stands in other areas of the Mediterranean Sea (Poulicek 1985, Sánchez-Moyano et al. 2000, Chemello and Milazzo 2002, Antoniadou and Chintiroglou 2005, Pitacco et al. 2014). The most species-rich family was Rissoidae, two species are exclusively associated with *C. amentacea* as well as two other species are

exclusively associated with *Cystoseira crinita*, no species of Rissoidae are exclusively associated with *Cystoseira compressa*. These species are micrograzers feeding preferentially on diatoms and epiphyte microalgae laying on *Cystoseira* leaves. Some other species are exclusively associated with only one or two algal associations as shown in the Table III-3.

The most frequent and top dominant mollusc species inhabiting *Cystoseira* associations along the coast of Ischia Island was the bivalve *Mytilus galloprovincialis* (96.6 % of the total abundance of all the individuals*)*. However only the juvenile stages (the most represented size ranges among the 0.3 – 3 mm) were strictly associated with algal canopy. The adult individuals were mostly found under the algal canopies attached to the hard rocky bottoms or among the holdfasts of macroalgae where they construct a real continuous barrier competing for the space with the above algal associations.

Except the presence of the mussels, it is possible to identify some differences in the pattern of association of molluscs community within the three algal assemblages, although the low level of dominance. In general the three algal species hosted diversified molluscs assemblages at the six sampling sites (Figure III-6) with low initial dominance and k-dominance curves reaching slowly the asymptote (Figure III-5 A-F). The gastropod *Scrissurella costata* is the most frequent and abundant species associated with *Cystoseira compressa*, this species is a deposit feeder feeding on food trapped in sediments retained by algal thalli. This association could be related to the high density of this algal species at all the analyzed sampling sites where it creates a tangled layer with its holdfast trapping high quantity of sediments. *Scissurella costata* was also the most frequent species associated with *Cystoseira crinita* at Scannella, this alga-animal association could be related to the peculiar habitat of rocky pool in which these species thrive. *Cystoseira crinita* in fact usually grows in the upper infralittoral zone on both low and intermediately exposed gently sloping rocky bottoms that are often subjected to a high degree of sedimentation (Sales and Ballesteros 2009, 2010). *Doto rosea* was the most frequent and abundant species associated with *Cystoseira amentacea*, this species is a specialized carnivore that commonly lives on hydroids, probably this species is more associated to the epibiontic hydroids living on *Cystoseira* surface rather than directly to the alga.

The highest dissimilarity in terms of molluscs composition was found among *C. compressa* and *C. crinita*, while *C. compressa* and *C. amentacea* are more similar.

The analyzed three algal associations only host juvenile mollusc stages, no adults were found. This confirm the importance of *Cystoseira* species associations as a nursery for molluscs recruitment.

No significant differences were found in the composition and the number of species at the different sampling sites, this let to suppose that the occurrence of the different zones within the marine protected area Regno di Nettuno does not influence the pattern of molluscs biodiversity associated with these algal species.

Comparing results from different areas is a challenging task because of the natural variability among geographic zones and the different sampling methods used. Although these difficulties, some similarities could be found in the number of mollusc species associated with upper infralittoral *Cystoseira* associations from other sites of the Mediterranean Sea. For example Chemello and Milazzo (2002) reported 35 species of molluscs associated with the species *Cystoseira barbatula* and *Cystoseira spinosa* at a shallow rocky shore at Lampedusa Island. Pitacco et al. (2014) reported 69 species of molluscs associated with the two algal sub-associations of *Cystoseiretum crinitae* and the association *Cystosereitum barbate* at the Gulf of Trieste. Çulha et al. (2010) found a total of 14 species associated with *Cystoseira barbata* faces at the Sinop Peninsula (southern Black Sea). Gozler et al. (2010) recorded 7 molluscs species associated with the species *Cystoseira barbata* at the southeastern Black Sea.

Although the dominance of the bivalve *Mytilus galloprovincialis* at all the analyzed sampling sites, the three species of *Cystoseira* are able to support diversified and structured molluscs assemblages. These results confirm the importance of *Cystoseira* associations in structuring habitat eligible for the mollusc assemblages especially during the juvenile stages.

These results must be taken as an incentive for a series of protection strategies towards these important habitat forming species since these are able to serve as a nursery and sheltered habitat supporting therefore a good level of associated biodiversity.

CHAPTER IV

CONCLUSIONS AND FUTURE PERSPECTIVES

IV.1 General conclusion

In the last decades, the understanding and the knowledge of the nature and space-time scale of biodiversity has developed greatly because the levels and pattern of biodiversity are being deeply modified by human activities. (Gaston 2010).

The terrestrial and marine biodiversity is decreasing at unprecedented rate as a result of the influence of anthropic impacts (Webb and Mindel 2015).

The attention has been focused on terrestrial and freshwater species extinction, the marine species have long been considered resilient to the extinction thus they have not been taken into account within extinction risk assessments (Webb and Mindel 2015).

For decades, the global oceans have coped with the impact of overexploitation, coastal transformation, habitat destruction. Often synergistic, these threats have degraded marine biodiversity with large and unpredictable impacts for the near future (Sala and Knowlton 2006).

The preservation of biodiversity therefore has become one of the most important challenge of conservation biology. In order to achieve this goal, a multidisciplinary approach taking into account the main components of biological diversity is fundamental.

As described in the introductive chapter of the present thesis, three different levels of biodiversity can be identified: species, genes and overall ecosystems.

Priorities and choices to monitor and manage all the aspects of biodiversity need to be evaluated. The choice of species to be preserved is strictly linked to the benefits that these species bring to the ecosystem.

Ecosystem engineering species are important for maintaining the health and stability of the environment where they live and where they are considered to be "*organisms that directly or indirectly modulate the availability of resources to other species by causing physical state changes in biotic or abiotic materials*" (Jones et al. 1994).

The macroalgae of the genus *Cystoseira* are considered important ecosystem engineering along the coasts of the Mediterranean Sea (Giaccone and Bruni 1973b, Ballesteros 1992).

Loss of Mediterranean *Cystoseira* species has been reported throughout the basin as a consequence of the habitat destruction, eutrophication, overgrazing by sea-urchins and fishes, leading to a shift to environments characterized by a lesser structural complexity (Cormaci and Furnari 1999, Thibaut et al. 2005, Airoldi et al. 2008, Falace et al. 2010, Sala et al. 2012b, Bianchi et al. 2014).

In the Gulf of Naples a recent study to outline the historical changes in macroalgal diversity highlighted a drastic decrease of *Cystoseira* species in the intertidal zones (Buia et al. 2013b, Grech et al. 2015).

The present study aimed to fill the gap of knowledge regarding the extent of diversity of macroalgae of the genus *Cystoseira* in the Gulf of Naples at different level of investigation:

- Species and population (Chapter II) by means of analysis of genetic variability of three *Cystoseira* species thriving in the intertidal shore;
- Community (Chapter III) through the analysis of malacofauna associated with three *Cystoseira* species along the coasts of Ischia Island.

Although the general trend of disappearance of these species from the Gulf of Naples, assemblages of two species, *Cystoseira compressa* and *Cystoseira amentacea* are still present along the intertidal shore of the coasts of Ischia Island, a third species *Cystoseira crinita* was only detected within a tide pool close to the sea surface.

The genetic analysis highlighted that the species *Cystoseira amentacea* and *Cystoseira crinita* are more variable in terms of polymorphic sites and number of haplotypes compared to *Cystoseira compressa*. This seems to be more related to the evolutionary history of these species rather than to their resilience towards the environmental conditions.

The analysis at community level highlighted the importance of *Cystoseira* species as nursery for the recruitment of molluscs since only juvenile stages were found. Although the dominance of the bivalve *Mytilus galloprovincialis*, it is possible to identify some differences in the pattern of association of molluscs community within the three algal assemblages. The malacofauna associated with *Cystoseira crinita* was more heterogeneous in terms of species composition. These results must be taken as an incentive for a series of protection strategies towards these important habitat forming species since these are able to serve as a nursery and sheltered habitat supporting therefore a good level of associated biodiversity.

Overall this study outline the importance of using a multi-approach in the analysis of diversity at different scales of investigation.

IV.2 Future perspectives

From a taxonomic point of view *Cystoseira* is one of the macroalgal genera that most requires a modern reassessment since it is considered under the process of active speciation (Roberts 1978).

As a future perspective additional molecular data based on a larger taxon sampling, including also the species distributed in the lower infralittoral zone of the Gulf of Naples, as well as the use of more preserved molecular markers, as the mitochondrial COX3

Cytochrome Oxidase subunit 3 gene or the mitochondrial mt23S could allow to clarify the remaining taxonomical doubts. Moreover the detailed geo-referenced distribution map of the species occurring in the upper infralittoral zone in the whole Gulf of Naples, performed by Grech D. within his Ph.D. project, could further expand the number of species and individuals.

To understand the population structure and variability of *Cystoseira* species, the study of genetic populations consisting mainly on the assessment of genetic structure and diversity (allelic and genotypic) is fundamental.

The microsatellites have proven to work properly for *Cystoseira amentacea* specimens, as a consequence for the future could be useful to expand the analyses to all the detected population all over the Gulf of Naples. Furthermore the development of additional microsatellites loci could be useful to infer studies on connectivity and population genetic at small to large spatial scales, and could provide essential insight for the development of conservation strategies for these important but threatened ecosystem engineering species.

Moreover the refinement of the genomic approach with RADSeq could be a very useful tool in the studies of population genetic and connectivity of these species.

Along the coasts of Ischia Island, the investigated *Cystoseira* associations however are distributed in a limited shallow area mainly subject to the impact of anthropogenic factors (Grech et al. 2015). Concerning the analyses of invertebrate community associated with these algae, it could be interesting to improve these results by long-term investigations in the near future in order to assess the changes at a time scale.

Moreover, investigations on other invertebrate taxa such as polychaetes and crustaceans would additionally clarify the importance of *Cystoseira* associations for benthic communities overall.

It could also be interesting and informative to collect the species *C. crinita* in other sites of the Tyrrhenian Sea in which this species co-exist with the other two species reported in the present study. This information will allow us to clarify if the differences within the algal structural complexity and in the diversity pattern of associated malacofauna are species-specific factors or are simply related to the peculiar habitat in which *C. crinita* thrive.

CHAPTER V

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Appendix 1

CTAB method for DNA extraction from brown algae

DNA isolation

1. Grind the tissue in a 1.5 ml tube with liquid nitrogen and a pellet-pestle until it becomes a fine powder
2. Add 700 µl of pre-warmed CTAB buffer and 10 µl of proteinase K (20 $mg \cdot mL^{-1}$) and vortex
3. Incubate at 60°C for 1 hour, mix by inversion every 10-15 minutes
4. Optional step: after incubating for 1 hour, add 30 µl RNAse A (10 $mg \cdot mL^{-1}$) and vortex. Incubate at 37°C for 30 minutes
5. Spin at full speed for 10 minutes
6. Transfer aqueous (upper) layer to a clean tube and add an equal volume of 24:1 chloroform:isoamyl alcohol and mix to emulsify
7. Spin at full speed for 10 minutes
8. Transfer aqueous (upper) layer to a clean tube

Precipitation

9. Add 50 µl of Sodium Acetate (NaAC) 3 M at RT to the tubes
10. Add 1 mL of absolute ethanol (EtOH) stored at -20°C
11. Mix gently few seconds to allow precipitation
12. Place the tubes at -80°C for 1 hour or at -20°C overnight
13. Centrifuge 20 minutes at 4°C at 14000 rpm
14. Quickly empty the tubes and leave the lids open

Ethanol Washing

15. Add 250 c of 70% EtOH stored at -20°C
16. Centrifuge the tubes 5 minutes at 14000 rpm at 4°C
17. Quickly empty the tubes and spin for few seconds to make sure all EtOH is going at the bottom
18. Place the tubes with the lid open under the fume hood to let EtOH evaporate
19. Resuspend the pellet in 100 µl high-salt TE and incubate at 60°C for 30 minutes

20. Add 5 µl of MagAttract Suspension G to each tube
21. Add 120 µl of absolute EtOH at RT
22. Mix gently to allow the DNA to adhere onto the surface beads
23. Leave at RT 5 minutes
24. Place the tubes on magnetic rack and pour off the EtOH

DNA Clean-Up

25. Wash the beads 3 times with 200 µl washing buffer and air-dry the beads 10 minutes at RT

DNA elution

26. Add 50 µl MilliQ distilled water to each sample well to resuspend DNA
27. Incubate at 60°C for 5 minutes and mix gently to allow the DNA bound to the beads to release into water
28. Place the tubes on magnetic rack and transfer DNA solution to new tubes

Appendix 2

TOPO TA Cloning® Protocol

TOPO TA Cloning® method allows the direct insertion of Taq polymerase-amplified PCR products into a plasmid vector. It takes advantage by the nontemplate-dependent terminal transferase activity of Taq polymerase that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Cloning

Reagents	**Volume**
Fresh PCR products	[10X the vector concentration]
TOPO® Vector [10 ng/µl]	0.5-1.5 µl
Salt solution	1 µl
H_2O	add to a total volume of 6 µl

1. Mix the reaction gently and leave at RT for 5 minutes.
2. Place the reaction on ice and proceed with the protocol for transforming competent cells

Transformation

1. Thaw on ice 1 vial of One Shot® TOP 10 chemically competent cells
2. Add 3 µl of the TOPO® Cloning reaction into a vial of One Shot® TOP 10 chemically competent cells and mix gently
3. Incubate on ice for 25 minutes
4. Heat-shock the cells for 30 seconds at 42°C without shaking
5. Immediately transfer the tubes on ice
6. Add 250 µl of room temperature LB medium
7. Cap the tube tightly and shake the tube horizontally for 1 hour at 37°C
8. Spread 30µl from each transformation on a prewarmed LB selective plate and incubate overnight at 37°C. Spread the remaining volume in a second plate to ensure that at least one plate will have well-spaced colonies
9. Pick between 10 to 30 colonies for the following analysis.

Appendix 3

Double Digestion RadSeq Protocol

Double Digest

1. Double digest 100-1000 ng of high quality genomic DNA with selected restriction enzymes, in a 20-50ul reaction volume.
2. Run the digestion as appropriate for the chosen REs. To ensure complete digestion, double digests were ran overnight at 37°C, then reactions were cooled to the room temperature before proceeding with the next step or alternatively stored at 4°C.
3. Clean the double digest with AMPure XP beads following the manufacturer's protocol,
4. Quantify the concentrations of the cleaned digests by BioAnalyzer and Qubit.

Anneal Adapters

Single-stranded oligos need to be annealed with their appropriate partner before ligation.

1. To create Adapter P1, combine each of the 48 oligos with its complementary in a 1:1 ratio in working strength annealing buffer (final buffer concentration 1x) for a total annealed adapter concentration of 40uM
2. To create common non-barcoded Adapter P2, combine the appropriate pairs of oligos
3. In a thermocyler, incubate at 97.5°C for 2.5 minutes, and then cool at a rate of not greater than 3°C per minute until the solution reaches a temperature of 21°C. Hold at 4°C.
4. Prepare final working strength concentrations of annealed adapters from this annealed stock

Adapter Ligation

1. Prepare a ligation mix as following:

Reagents	Volume for 1 reaction
P2 adapter	0.5µL
Buffer T4 ligase [10X]	6 µL
T4 ligase [400U/µL]	0.4 µL
H_2O	13.1 µL

2. Distribute 20 µL of ligation MIX per tube/well + 5 µL of P1 adaptor [4 µM] +35 µL of double digested DNA
3. Incubate at room temperature for 6 hours.

PCR Amplification to Generate Illumina Sequencing Libraries

To add Illumina flowcell annealing sequences, multiplexing indices and sequencing primer annealing regions to all fragments and to increase concentrations of sequencing libraries, a PCR amplification was performed with specific primer for adapters.
Combine the completed reactions and clean with AMPure XP beads
Run cleaned PCR samples on an Agilent Bioanalyzer to quantify molarity and library fragment size distribution. A secondary quantification such as fluorometer (Invitrogen Qubit) or qPCR is also recommended.

Pooling prior to size selection

Samples individually barcoded with a unique P1 adapter were pooled after the ligation step and cleaned with AMPure XP beads.

Size Selection with Sage Science Pippin-Prep

Automated DNA size selection was used to recover fragment sizes appropriate for Illumina sequencing.

Appendix 4

PERMANOVA results of pair-wise t-tests applied on macroalgal features

PERMANOVA results of pair-wise t-tests applied on macroalgal features for the interaction alga x site for pair of levels of factor alga. t values by 9999 permutation. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not-significant. *a: t test between C. compressa and C. amentacea, b: t test between C. compressa / C. crinita, c: t test between C. amentacea / C. crinita*.

	t-values					
Sites	**CA**	**SP**	**SA**	**SC**	**PI**	**PC**
Density	1.45 ns	0.15 ns	0.97 ns	1.55_a ns 2.06_b ns 0.75_c ns	0.17 ns	3.03 **
Biomass	1.14 ns	0.72 ns	0.42 ns	0.38_a ns 0.34_b ns 0.01_c ns	1.98 *	1.64 ns
Height	0.52 ns	2.56 *	0.98 ns	2.06_a ns 4.43_b ** 2.41_c *	3.59 **	5.13 **

Appendix 5

PERMANOVA results of pair-wise t-tests applied on diversity index

PERMANOVA results of pair-wise t-tests applied on diversity index for the interaction alga x site for pair of levels of factor alga. Exp H': exponential Shannon, 1/Simpson: reciprocal Simpson. t values by 9999 permutation. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not-significant. *a: t test between C. compressa / C. amentacea, b: t test between C. compressa / C. crinita, c: t test between C. amentacea / C. crinita*.

	t-values											
Sites	CA		SP		SA		SC		PI		PC	
Nr. species	0.35	ns	2.29	ns	0.86	ns	1.04_a	ns	0.33	ns	1.46	ns
							2.80_b	*				
							1.97_c	ns				
Nr. individuals	1.03	ns	2.36	ns	1.74	ns	0.75_a	ns	1.22	ns	0.44	**
							0.91_b	ns				
							0.18_c	ns				
Exp H'	0.33	ns	1.20	ns	0.38	ns	0.75_a	ns	0.40	ns	1.98	ns
							2.50_b	*				
							2.06_c	ns				
1/Simpson	0.46	ns	0.60	ns	0.24	ns	0.18_a	ns	0.82	ns	2.18	ns
							1.99_b	**				
							1.92_c	*				