Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Biodiversità ed Evoluzione

Ciclo 28

Settore Concorsuale di afferenza: 05/C1

Settore Scientifico disciplinare: BIO/07

GENETIC AND MORPHOLOGICAL FEATURES OF PATELLA CAERULEA AND PATELLA RUSTICA ACROSS MEDITERRANEAN MARINE PROTECTED AREAS

Presentata da: **Patricia Martí Puig**

Coordinatore Dottorato **Relatore**

Barbara Mantovani **Laura Airoldi**

Esame finale anno 2016

Università di Bologna - Centro Interdipartimentale di Ricerca per le Scienze Ambientali

Academic Year: 2013-2016

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Submitted by

Patricia Martí Puig

Academic advisors: Prof. Marco Abbiati, Dr. Massimo Ponti

This dissertation is submitted for the degree in

Doctor of Philosophy in Biodiversity and Evolution (University of Bologna)

March 2016

Within the Training Network for Monitoring Mediterranean Marine Protected Areas (MMMPA, funded by the European Commission within the 7th Framework Programme (Call FP7-PEOPLE-2011-ITN, Grant Agreement no.: 290056)

Cover picture

Patella rustica (left) and *Patella caerulea* (right) at Portofino MPA. Photo by Patricia Marti-Puig

This thesis is dedicated to my nephew Matteo, the best present during my PhD.

'PROTECT AND CONNECT THE OCEANS

DON'T LEAVE MPAs ALONE!'

http://m.youtube.com/watch?v=bFhexhq6tGE

ACKNOWLEDGEMENTS

I would like to acknowledge first to Prof. Marco Abbiati, head of Experimental Applied Ecology department, who accepted me in his research group as a PhD student and followed me during these years. To Dr. Massimo Ponti, partner coordinator of the project MMMPA, for being always available to help, giving me advice and teaching me to be a better researcher, during all the process of my PhD. Of course, thanks to Dr. Federica Costantini and Dr. Adriana Villamor for their advice and assistance with the genetic analysis, for their patience and perseverance. Thank you to the MPA managers who allowed the sampling for this work. To Prof. José Charton and his students (José Pereguiñez and Ramón García), to Ubica guys (Ubaldo Pantaleo and Marco Palma) and to the MPA manager of Tavolara Augusto Navone for their assistance in the field-work. To Roberto Buonomo for his assistance in R programing. To Dr. Paolo Albano, Dr. Ayesha Tulloch and Dr. Francine Keshaw for their optimism and helpful collaboration in the applications of my PhD research. Thanks to my other genetic colleagues from Bologna, specially Alice, Greg & Carlo for the coffee times and for their advices.

I would like to specially thank to all the MMMPA dream team, for the fruitful collaboration during project and friendship, especially to the MMMPA Italian girls (Vessa and Paula) for the beautiful short-movie (Zoe and her adventures in the sea) that we create together and to Dani for "the psychology and life lessons". To Carlo Cerrano, the coordinator or the project, for his huge effort coordinating the MMMPA team. Thanks to all the teachers and trainers that help me to develop important skills for my PhD, life and future career.

I would like to take this opportunity to thank my family and loved ones. To my special girl friends: Veronica, Vera and Fatine for the "gossip" times. To my tango, salsa, musician and CS friends: without you my life in Ravenna would not have been the same! To Maria Crespo, my travel partner, who helped to disconnect from research. To my forever friends in Barcelona (Belen, Marc, Nadia…), who always supported me, specially to Sergio, Ivonne, Berta and

Carlitos for visiting me! Thanks also Ivonne Peña for your very useful advises as an experience Doctor. To Jorge Aguadero for his leadership inspiration and friendship. Special thanks to my beloved Dawid, mum, sister and nephew for their support, love and inspiration! “Mami, gracias por los bocadillos de jamón y las comidas de madre mientras preparaba la tesis. ¡Madre solo hay una! Nina, gracias por los chocolates y paquetes que me enviabas durante la tesis”.

Finally, I would like to thank to “Marie Curie”, the grant that offered me the opportunity for doing this research. During the last three years as a PhD student, it was an intense professional and personal learning process, which has opened the doors for continuing and growing in my career.

Patricia Martí Puig

29-February-2016

“The MMMPA dream team”

AUTHOR’S DECLARATION

The research for this thesis was supported by funding from the European Union within the Training Network for Monitoring Mediterranean Marine Protected Areas (MMMPA), a Marie Curie Initial Training Network, funded by the European Commission within the 7th Framework Programme (Call FP7-PEOPLE-2011-ITN, Grant Agreement no.: 290056).

All chapters in this thesis were written by Patricia Marti-Puig and are formatted in the style of manuscripts for publication. Comments and editing were provided by Dr. Massimo Ponti, Dr. Federica Costantini and Prof. Marco Abbiatti. No data can be taken out of this work without prior approval of the thesis tutor/s. I hereby confirm that I have independently composed this PhD thesis and that no other than the indicated aid and sources have been used. This work has not been presented to any other examination board.

Patricia Martí Puig

Ravenna, 26-March-2016

The “connectivity problem”

“I am nothing. I’m like someone who’s been thrown into the ocean at night, floating all alone. I reach out, but no one is there. I call out, but no one answers. I have no connection to anything.”

— Haruki Murakami, 1Q8

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Abstract

Marine Protected Areas (MPAs) were initially created to protect the living, non-living, cultural and/or historical values from human activities. The Convention on Biological Diversity (CBD; Earth Summit in Rio de Janeiro on 5 June 1992) has set a target of protecting 10% of the coastal and marine areas by 2020, which has led to a rapid increase in the creation of MPAs worldwide. Within this context, there is a growing concern regarding the number of efficient MPAs. One of the main issues is that biological or ecological features of marine species as well as ecosystem processes are not taken into account in MPA design. Deciding criteria for species management requires considerable information collected from a number of sources, including morphometric data, genetic data and distributional data. Morphometric tools are useful to study species taxonomy, or to provide information about the morphological variability, size and growth of the species, which is essential for MPA monitoring. Genetic tools can be use to resolve species taxonomy or population structure, allowing to estimate genetic diversity and connectivity of populations at different temporal and spatial scales. Both morphometric and genetic data used in combination provide a powerful tool that should be considered in MPA assessment. However, the accurate interpretation and the integration of this information into marine spatial planning is specially challenging. The aim of this PhD thesis was to develop a protocol for monitoring Marine Protected Areas by studying the morphology and genetics of two closely limpet species (*Patella rustica* and *Patella caerulea*) across MPAs in the Western Mediterranean sea. Overall, the results of this thesis provides support the inclusion of the morphological and genetic tools into management plans, and in the guidelines for the monitoring to improve and/or maintain MPA health and effectiveness.

PhD thesis outline

In Chapter I, an overall Introduction of the work is provided, raising the questions: 1) MPAs are well design? 2) Why is important to integrate morphometric and genetics tools for MPA design? 3) How do we identify and integrate these tools in MPA design? At the end of the Chapter, I provided the project context and aims of my PhD thesis.

In Chapter II a review of the literature and state of the art on genetic connectivity in benthic invertebrates in temperate MPAs was carried out. In this chapter a conceptual framework for planning effective studies on genetic connectivity in MPAs network, including general recommendations on sampling design, key species and molecular markers to use, were provided. I highlighted the importance of a sampling design that includes protected and non-protected sites, considering several species and different markers depending on the temporal and spatial scales needed. The content of this chapter has been published in Advances in Oceanography and Limnology (Marti-Puig et al., 2013).

In Chapter III, genetic and morphometrics of the two selected sympatric species *Patella caerulea* and *P. rustica*, were investigated in the chosen locations, which include MPAs and adjacent areas. The aim was to compare morphological variability across sites and between species. A combination of genetic and morphometric characters could clearly differentiate the species. Morphometric methods detected a high morphological variability in *Patella caerulea* within sites. Chapter II highlights the importance of combining morphometric and genetic tools for MPA monitoring. The preliminary results of this study were presented, as a poster, in the ECSA56 conference (ANNEX) in Sesimbra (Portugal).

In Chapter IV, levels of genetic diversity and structuring of *P. caerulea* and *P. rustica* among and within four MPAs in the western Mediterranean Sea have been investigated using a multifactorial hierarchical sampling design. For this purpose, *Patella* populations were sampled in replicated sites inside and outside MPAs and analysed using mitochondrial and nuclear markers. This work aimed to answer specific questions: 1) are there significant differences in genetic variability inside and outside MPAs?; 2) is there a significant genetic structuring among populations across MPAs and within them?; 3) are the genetic features of the two

species comparable? Mitochondrial marker showed high genetic connectivity over long term at Mediterranean scale, suggesting that western Mediterranean MPAs could be studied as a single management unit. Microsatellite DNA revealed detailed patterns on the genetic structure at MPA scale variable between species and sites. The results from this chapter put in evidence the importance to use multi-species and multi-scale approaches for the study of genetic diversity and connectivity.

In Chapter V, I provide the guidelines for MPA design criteria, integrating the results obtained in Chapter III and the results obtained by another partner of the MMMPA project working on fish connectivity. Data generated by all the Work Packages of the MMMPA project were integrated in the guidelines for MPA design criteria and for the establishment of a coherent network of MPAs in the Mediterranean. These guidelines were delivered to the European Union (Marti-Puig, 2016). The results from Chapter V highlight the importance of genetic diversity and connectivity studies for the integration into MPA management.

The outcomes in communication and outreach (e.g. creation of a short animation movie, conference presentations…) carried out during the MMMPA project are included in the ANNEX.

CHAPTER I. GENERAL INTRODUCTION

Marine Protected Area of Tavolara, Sardegna, Italy. Photo source: Patricia Marti-Puig

1.1. Marine Protected Areas (MPAs): are they well designed?

Due to the anthropogenic impacts on the ocean during the lasts decades, marine resources are becoming more and more overexploited. An increase in human activities and population expansion towards the coast has caused a rapid degradation of the ocean's functions and biodiversity (Lubchenco et al., 2003). Several studies demonstrate that fishes and other exploited marine populations have collapsed (Hutchings and Reynolds, 2004; Halpern et al., 2008) and there is an increasing need to protect and preserve the marine habitats and their resources.

Marine Protected Areas (MPAs) were initially created to protect the living, non-living, cultural and/or historic heritage from human activities. Nowadays, MPAs are the hope to preserve marine biodiversity and ecosystem processes. A MPA is defined as "any area of intertidal or subtidal terrain, together with its overlying water and associated flora, fauna, historical and cultural features, which has been reserved by law or other effective means to protect part or all of the enclosed environment" (Kelleher, 1996). MPAs have an important effect preserving the biomass of fishes and other marine fauna and flora, thus contributing by the spill over effct to the or dissemination of larvae and adults outside the reserve (Polunin and Roberts, 1993; Russ, 2002).

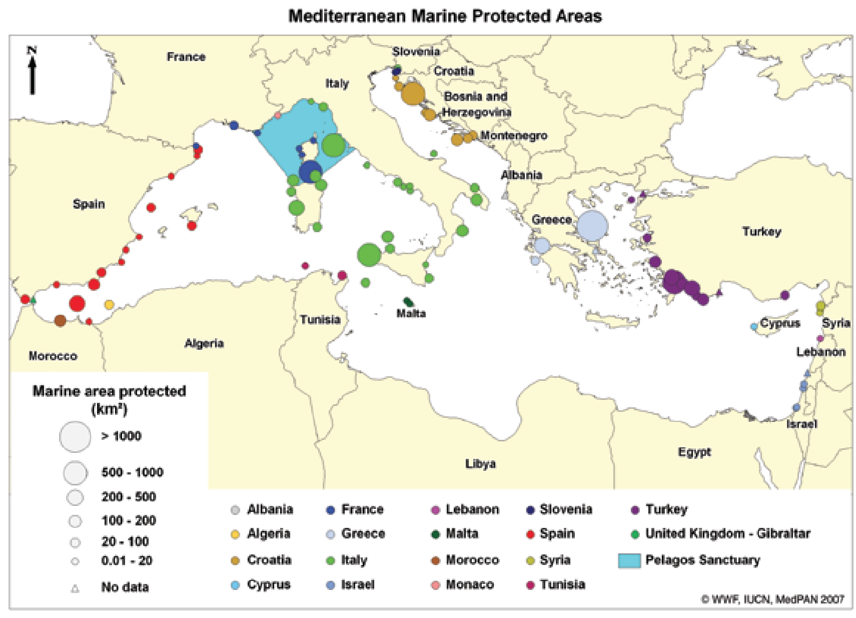


Figure 1.1. Distribution of Mediterranean MPAs. Relative size of each MPA is shown according to different class sizes. Source: Abdulla et al., 2009

The Convention on Biological Diversity (CBD; Earth Summit in Rio de Janeiro on 5 June 1992) has set to protect the 10% of the coastal and marine areas by 2020, which has led to a rapid increase in the creation of MPAs worldwide. However, we are still far to reach CBD target. For example, in the Mediterranean Sea, the marine protected and managed areas cover only the 4% of the entire basin, 0.4% excluding the Pelagos Sanctuary (Figure 1.1). Within this context, there is a growing concern regarding the number of effective MPAs (Agardy, 1994; Babcock et al., 2010; Edgar et al., 2014), that should be able to maintain marine ecosystem functioning and preserve marine species, or if they are just “paper MPAs”.

One of the main issues about MPAs planning and establishment is that they are not usually designated taking into account the biology and ecology of the marine species, and their habitats. MPAs are usually designed and managed as isolated units, which is not enough to ensure the resilience of marine ecosystems, since most marine species are arranged in metapopulations, connected by the movement of adults or larvae (Kaunda-Arara and Rose, 2004; Starr et al., 2004). The selection of the target (e.g. species, habitat, processes) in planning MPA design are very relevant, as each organism is unique in terms of biology and ecology. Size, locations and spatial arrangement of MPAs is depending on the species, set of species or area of study. Marine organisms have a wide range larval or adult dispersal distances that can vary depending on the species from meters to 1000km (Palumbi, 2003; Coleman et al., 2011), influencing their distribution, genetic structure and connectivity patterns (Fig 1.3.; Coleman et al., 2011, Toonen et al., 2011; Berumen et al., 2012). Well-connected populations have the potential to enhance the persistence of marine species, contributing to stabilize ecosystems processes. Export of larvae from source population that can help the recovery sink populations impacted by disturbances (Hastings and Botsford, 2006). Design of Marine Protected Areas requires an understanding of larval transport in and out, whether these areas will be self-seeding, whether they will import recruits from surrounding exploited areas, and whether they can exchange recruits to other areas (Palumbi, 2003). Moreover, MPAs are generally not designed to protect genetic diversity, which is essential for the long term viability of marine populations, by allowing populations to preserve their adaptive and evolutionary potential (Bernhardt and Leslie, 2013). Finally, the ability of species and ecosystems to adapt to changing conditions, is an essential

component of ecological resilience. Adaptive capacity will depend on the phenotypic plasticity, dispersal and evolutionary genetic change (Williams, 2008; Hoffmann and Sgrò, 2011). All these components are central for MPA assessment and conservation management strategies, helping to define units of conservation for designing future Marine Protected Areas and ensure resilience of populations (Kritzer and Sale, 2004; Pineda et al., 2007; Jones et al., 2009, Gaines et al., 2010).

1.2. Morphometric and population genetics in MPA design

Deciding location, size and boundaries for protection of marine species requires considerable information collected from a number of sources, including morphometric data, genetic data and distributional data. New morphometric and genetic tools are now available providing several advantages in analysing species distribution and population connectivity, specially since they have become more efficient and non-destructive, allowing their application on endangered species and focal species (Calò et al., 2013, Marti-Puig et al., 2013; Csencsics et al., 2010).

Morphometric tools are useful to study species taxonomy, or to provide information about the morphological variability, size and growth within species, which is essential for MPA monitoring. Morphometrics implies quantitative measurement and analysis of morphological traits, such as size and shape, which has traditionally been accomplished using manual linear measures. Nowadays, morphometric methods using digital images offer a new quick and precise tool to analyse morphometric traits (MacLeod et al., 2000; Rohlf and Marcus, 1993). These powerful methods offer several advantages rather than traditional methods, by facilitating better data collection, more effective descriptions of shape, and new analytical tools (Cadrin and Friedland, 1999, Bookstein, 1997).

Genetic tools can be use to study species taxonomy or the population structure, providing unique information for marine protection, management, and spatial planning at different temporal and spatial scales (Pelc et al., 2009). These tools allow estimating genetic diversity of the populations and their connectivity by assessing changes and differences in the frequency of the genes (Hedgecock et al., 2007). Genetic tools have been applied for the evaluation of population

connectivity and diversity in a wide variety of marine taxa. Different genetic markers can be used to estimate genetic structure and connectivity of populations, depending on the species and the temporal or spatial scale of interest (Selkoe and Toonen, 2006).

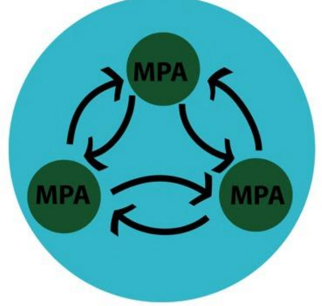
Morphometric and genetic tools can be combined to disentangle species or populations, when species identification is challenging, e.g. in the case of some invertebrate species and fish species (Cadrin and Friedland, 1999). The use of genetic and morphological data also allows the interpretation of patterns of variability, enabling the investigation of the source of a possible inter-population variation (Silva et al., 2010).

1.3. Integrating morphometric and genetic data in MPA design

Morphometry can be measured using traditional manual methods and digital methods (such as landmark or outline methods). Digital morphometric methods offer the advantage of capturing differences in structures that are not easily observed by traditional types of measurements. Size and shape of the organisms can be detected by a semi-automatic identification of the outline of the organism. Outline methods use empirical functions to represent coordinates of outline shape (Rohlf and Marcus, 1993). The most common outline method involves fitting a Fourier series to the point coordinates along the perimeter of a morphometric feature (Kaesler and Waters, 1972). Fourier Shape Descriptors (EFDs) are commonly used as multivariate observations for discriminant analysis, and several studies have successfully used these methods for analysing different organisms, such as fishes (otoliths shape) and marine invertebrates (reviewed by Friedland, 1996). Several descriptors related to the identification of the species, of growth patterns, size and shape, can be used in population monitoring for MPA management (e.g. González-Wangüemert et al., 2014, Silva et al., 2010).

Genetic markers and indexes allowing the description of the populations genetic structure and patterns could be used and integrated into MPA design (Figure 1.2). Mitochondrial DNA (mtDNA), with slow rates of evolution, provides information at large spatial and long temporal scales, therefore, it can be used to define genetically distinct populations or marine management units at scale of networks (Beger et al., 2014). In contrast, microsatellite markers, with high

mutation rates and polymorphism, provide information at local spatial and short temporal scales (Cornuet and Luikart, 1996; Petit et al., 1998; Kalinowski, 2004). Combining the information obtained by several markers should provide insight evolutionary and contemporary scales to understand the processes that have determine the actual genetic structure of the populations



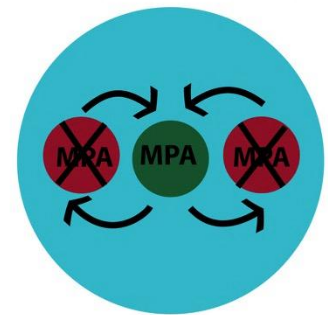


Figure 1.2. Different markers (mitochondrial and nuclear markers) and their utility in estimating connectivity depending on the spatial and temporal scales of analysis.

The most common metrics that can be obtained are genetic diversity indexes (haplotype diversity, nucleotide diversity, allelic richness, private allelic richness) or genetic structure and connectivity indexes (F_{ST}, D_{JOST}, migration rate) (Lowe and Allendorf, 2010). The most commonly reported estimates of DNA sequence diversity are haplotype and nucleotide diversity. Haplotype diversity represents the probability that two randomly chosen haplotypes are different (haplotype diversity, h; Nei, 1987), while nucleotide diversity represents the average number of nucleotide differences per site between two randomly chosen DNA sequences (nucleotide diversity, π; Nei and Li, 1979). Allelic richness represents the number of observed alleles and their frequency distribution within a population standardized for sample size, while private alleles is the number of alleles that are unique from that population. The higher the allelic richness the more variable the population is, while the higher private alleles the more unique the population is considered to be. F_{ST} is a genetic metric used to estimate genetic differentiation between populations, while migration rate measures how the populations are connected, estimating the number of migrants per generation exchanged between them.

All these tools provide important information about the morphology, and genetic structure of the populations that should be considered for the MPA design. However, the accurate interpretation and the integration of this information into marine spatial planning is specially challenging (Putman and Carbone, 2014). Moreover, there is a need to develop a protocol able to communicate, in a comprehensible way, this information to marine policy and management communities.

1.4. Project context and aims

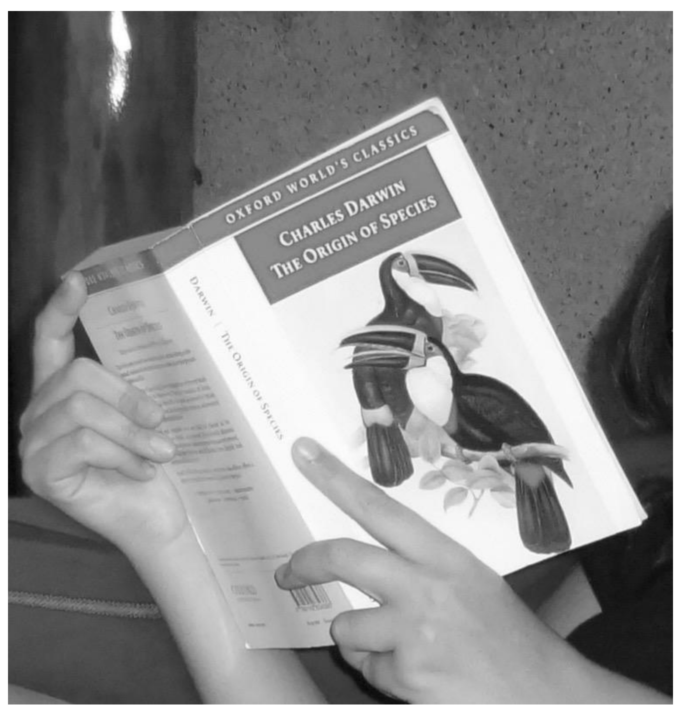
My PhD project was developed within the European funded project, the training network for Monitoring Mediterranean Marine Protected Areas (MMMPA; FP7-PEOPLE-2011-ITN). The aims of the MMMPA project were to train a new generation of MPA scientists and managers, equipping them with a flexible set of skills essential within a wide range of professional environments, including public administration, local authorities, industry and academia, and to improve the methods for assessing the current status of MPAs in the Mediterranean Sea. To reach this goal, the project was divided in work packages (WP) to study biodiversity assessment and ecosystem functioning (WP1), local fisheries description and management (WP2), biodiversity threats (WP3), socio-economic assessment (WP4) and integrated coastal zone management (WP5). At the end of the project guidelines on the application of innovative MPA monitoring approaches were delivered to the European Union to the Mediterranean MPA managers. My PhD project was within the WP3 (WP 3.1 - Connectivity among populations of the genus *Patella* among Mediterranean Marine Protected Areas).

The aim of this PhD thesis was to develop a protocol to include morphometry and population genetics in monitoring of Marine Protected Areas. The study focussed on two closely related limpet species (*Patella caerulea* Linnaeus, 1758 and *Patella rustica* Linnaeus, 1758) that were analysed across 4 MPAs in the Western Mediterranean sea. *Patella* species were used as key species because: a) they have a widespread distribution and are relatively easy to collect, b) they reproduce by planktonic larvae that can be spread over long distances by the oceanographic currents (Ribeiro, 2008), c) they play a key ecological role in intertidal habitat (Arrontes et al., 2004, Guerra and Gaudencio, 1986); d) they are vulnerable to anthropogenic pressures, including trampling, human harvesting,

and climate change (Guerra-Garcıa et al., 2004) e) there are available genetic markers and previous studies in the Mediterranean provide a background on the population structure of the species (Pérez et al., 2007; Sá- Pinto et al., 2010, Villamor A, 2014, Fauvelot et al., 2009) f) as other marine invertebrates they show a high morphological variability, challenging species identification and monitoring (Mauro et al., 2003).

Overall, this thesis provides a guideline to include morphology and genetics into specific monitoring and management plans that will improve and/or maintain MPA health and effectiveness.

Chapter II: RESEARCH BIBLIOGRAPHY AND MARINE PROTECTED AREA SAMPLING DESIGN CRITERIA



Publication note: The content of the following chapter has been published: *Patricia Marti-Puig, Federica Costantini, Luca Rugiu, Massimo Ponti & Marco Abbiati (2013) Patterns of genetic connectivity in invertebrates of temperate MPA networks, Advances in Oceanography and Limnology, 4:2, 138-149, DOI: 10.1080/19475721.2013.850445*

2.1. Abstract

Temperate reefs are among the most threatened marine habitats due to impacts caused by high density of human settlements, coastal development, pollution, fisheries and tourism. Networks of marine protected areas (MPAs) are an important tool for ensuring long-term health and conservation of ecological processes in the marine environment. Design of the MPA network has to be based on deep understanding of spatial patterns of species distribution, and on the make-up of connectivity among populations. Most benthic invertebrates are sessile and/or sedentary in the adult phase, and their dispersal relies mainly on the gametes and/or larval behaviours. Genetic markers allow us to quantify gene flow and structuring among populations, and to infer patterns of genetic connectivity. Based on the information available in the peer-reviewed literature on genetic connectivity in benthic invertebrates of temperate MPAs, we provide a comment about the gaps and the needs. Moreover, we propose a rationale to plan and optimise future studies on this topic. A conceptual framework for planning effective studies on genetic connectivity in an MPAs network is provided, including general recommendations on sampling design, key species and molecular markers to use.

Keywords: marine protected areas; temperate biogenic reefs; sampling design; molecular markers; population genetics

2.2. Introduction

Temperate reefs are primary or secondary hard substrata, which include rocky bottoms, vertical cliffs and a variety of biogenic structures (e.g. oysters and mussels beds, vermetid and sabellarid reefs, trottoire, coralligenous rims and banks, deep-sea corals), located in areas with a temperate climate and subjected to a relatively moderate seasonal changes Spalding et al., 2007. Temperate reefs support some of the most productive and diverse assemblages Suchanek, 1994 providing habitat, feeding grounds, recruitment and nursery areas for a variety of invertebrate and vertebrate species. Subtidal biogenic reefs also are hot spots of biodiversity in many temperate seas (e.g. Mediterranean Sea (Coma et al., 2006; Coll et al., 2010, temperate Australian waters (Wernberg et al., 2011). Temperate coastal habitats, similarly to the tropical ones (Roberts et al., 2002; Hughes et al., 2003; Bellwood et al., 2004), host the majority of world's human population, and are among the most threatened habitats globally due to density of human settlements, coastal development, pollution, fisheries and tourism (Airoldi and Beck, 2007; Lotze et al., 2011). However, understanding of the ecological processes and functioning of temperate marine habitats, as well as their conservation status, is inadequate (Kennish, 2002; Steneck et al., 2002; Thompson et al., 2002; Airoldi and Beck, 2007; Lotze et al., 2011).

The International Union for Conservation of Nature (IUCN) defines the Marine Protected Areas (MPAs) as a "clearly defined geographical space, recognized, dedicated and managed, through legal or other effective means, to achieve the long-term conservation of nature with associated ecosystem services and cultural values". As a rule, they are designed to reduce anthropogenic impacts that nowadays threat the entire marine realm (Airoldi and Beck, 2007; Baskett et al., 2007; Gaines et al., 2010; Lotze et al., 2011). MPAs are considered an effective tool to preserve and restore habitats and biodiversity, re-establish over-harvested marine resources and to manage fisheries (Claudet et al., 2008; Claudet et al., 2010; Gaines et al., 2010; Coleman et al., 2011). Although to date about 7,000 MPAs have been established worldwide, less than 2% of the world oceans and seas are currently protected and only a small proportion of MPAs are located in temperate areas (source http://www.mpatlas.org/). Moreover, MPAs are usually located in coastal or insular areas, and only recently attempts to establish

MPAs in the pelagic domain have been made (Guidetti et al., 2013). Networks of MPAs are widely acknowledged to be an important tool for ensuring the long-term health and conservation of ecological processes in the marine environment (Lubchenco et al., 2003). To correctly design a network of MPAs a deep understanding of spatial patterns of species distribution, focusing on endangered and threatened taxa (Sala et al., 2002; Roberts et al., 2003), and on the makeup of genetic connectivity among populations is needed.

The term connectivity has been recently introduced in marine ecology (Cowen et al., 2000), being related to marine populations. A formal definition of this term has been provided in 2002, in the special issue "Open vs. Closed Marine Populations - Synthesis and Analysis of the Evidence" of the Bulletin of Marine Science: "the degree to which local larval production results in recruitment to other populations" (Warner and Cowen, 2002). Investigating levels of connectivity (evolutionary and demographic) allow to: a) define ranges of effective larval dispersal (Villamor A, 2014) b) understand the supply of larvae and adults into and out of a MPA (Palumbi, 2003) c) quantify gene flow and assess levels of genetic diversity. Connectivity ensures the long-term persistence and resilience of populations under current and future scenarios of anthropogenic change (Kaplan et al., 2009; Planes et al., 2009). Different methods can be used to measure connectivity, and each method is best suited to address different ranges of spatial and temporal scales of variation (Jones et al., 2009). Direct methods, such as visual observations and mark-recapture, provide the most accurate information on animal movement over demographic time-scales, but not over evolutionary time scales (Kool et al., 2013). Moreover, direct methods are affected by small spatial or seasonal variations in recruitment that may not be informative over larger spatial or temporal horizons. The majority of the literature on this topic analyses the importance of connectivity in MPAs network based on commercial and non-commercial fishes and corals (Charton et al., 2000; Crooks, 2006; Cowen and Sponaugle, 2009; Kool et al., 2013). However, benthic invertebrates, which include several habitat formers and ecosystem engineers (e.g. oysters, mussels, polychaetes, bryozoans, corals), are important species in temperate reefs habitats since they are conducive to the establishment of the rich species assemblages. Keystone predators and eroding taxa are shaping the morphology and dynamics of these habitats (Piraino et al., 2002). Benthic invertebrates usually have sessile or sedentary adult phase, and their dispersal relies mainly on the gametes and/or

on larval behaviours. However, for these species it is not always possible to use direct method to measure connectivity patterns due to the difficulties in tracking the larvae in the field, therefore indirect approaches are needed.

Genetic markers, able to discriminate the spatial scales at which populations can be differentiated into discrete units, allow us inferring on patterns of genetic connectivity. Genetic connectivity represents the degree to which gene flow affects evolutionary processes among populations, and genetic structure estimators provide a proxy of the number of larvae migrating between populations. Recent studies have shown that larval pelagic duration could be poorly correlated with genetic structure, which is recording the effective migration between populations (Kelly and Palumbi, 2010). Gene flow is affected by other factors, such as larval ecology, life history of the species, stochastic processes in population and oceanographic conditions (Weersing and Toonen, 2009; Marshall et al., 2010; Selkoe et al., 2010; Jaquiéry et al., 2011; Basterretxea et al., 2012; Sundelof and Jonsson, 2012). Genetic connectivity can be estimated by different tools and genetic markers (e.g. allozymes, mitochondrial DNA, nuclear DNA). Effectiveness of the genetic tools may vary, depending on the species and on the spatial and temporal scales of interest (Hellberg et al., 2002; Berumen et al., 2012).

The aim of this work is to summarize the information available in the literature on genetic connectivity in temperate MPAs with a focus on marine invertebrates. A survey of the scientific literature has been done based on species, molecular markers, and spatial scales. Moreover, the gaps in available information and possible approaches to address these gaps were discussed. Criteria for planning effective studies to analyse genetic connectivity in MPAs network are discussed, including the importance of larval connections within an MPA, among MPAs, and between MPAs and surrounding non-protected areas (Jones et al., 2009).

2.3. Literature review

Peer-reviewed literature on MPAs available in ISI Web of Science was selected using the following key words as a topic: genet* AND connectivit* AND ("marine reserve*" OR MPA* OR "marine protected area*"). The searches resulted in 115 hits, published from 2002 to 2012 (Figure 2.1).

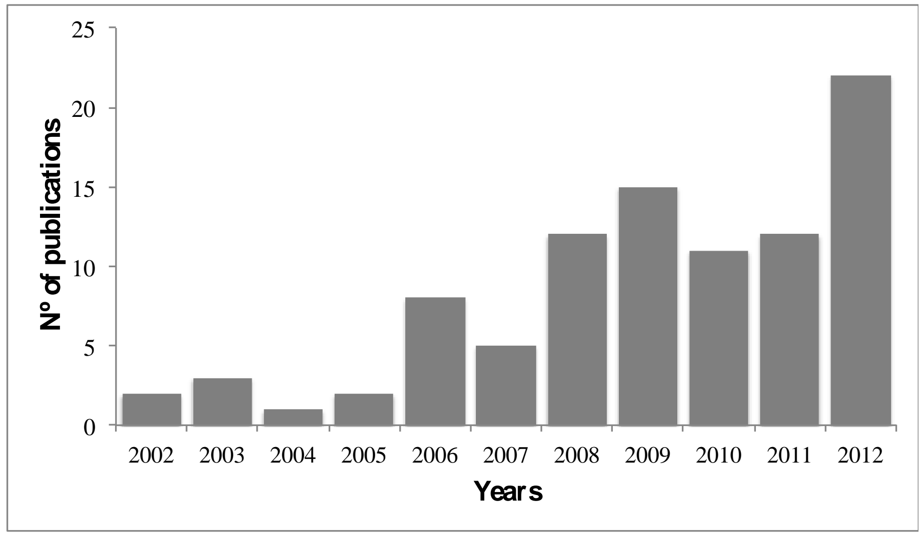


Figure 2.1: Number of publications found: in ISI Web of Science using the key words as a topic: genet* AND connectivit* AND ("marine reserve*" OR MPA* OR "marine protected area*") per years.

No publications were found before the 2002, the year when the term connectivity has been formally defined. Selected papers were filtered manually, and among the 115 studies, 93 were related to the topic addressed. Out of the 93, twelve were reviews, and therefore discarded, and 3 were discarded because dealing with deep-sea habitats. From the 78 papers left, 53 were dealing with tropical MPAs and only 25 were on temperate MPAs (Figure 2.2).

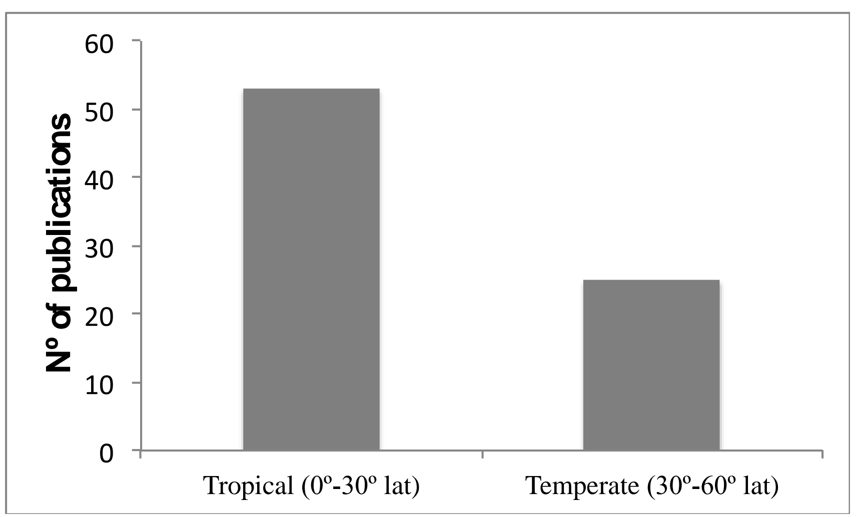


Figure 2.2: Number of publications comparing tropical and temperate areas.

Studies on temperate habitats included 10 dealing with fishes, 13 dealing with invertebrates, 1 on mammals, and 1 on algae (Figure 2.3).

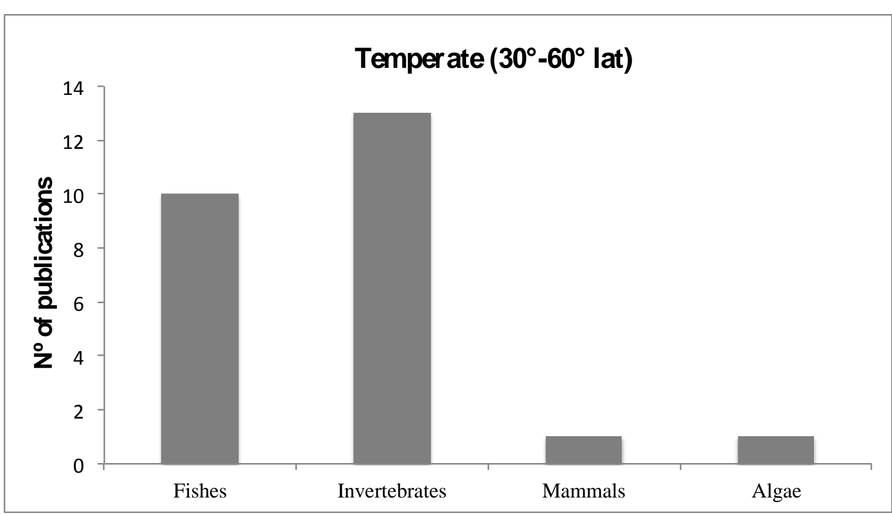


Figure 2.3: Number of publications found in temperate habitats on fishes, invertebrates, mammals and algae

The most investigated benthic invertebrate Phylum in temperate habitats were Mollusca, while studies on other Phyla (e.g. Cnidaria, Arthropoda and Annelida) were less frequent (Figure 2.4).

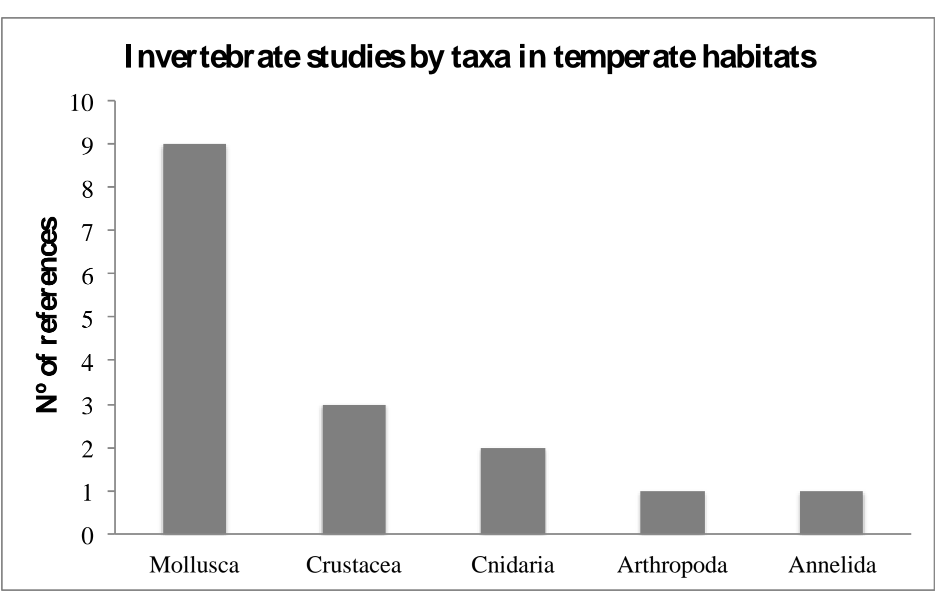


Figure 2.4. Number of publications found on benthic invertebrates by *Phyla* in temperate habitats.

The literature review showed that most of the 13 papers on marine invertebrates stressed the importance of studying genetic connectivity to improve MPAs design and effectiveness, but only 3 of them where specifically focused on the assessment of effectiveness of MPAs. One of them was focused on MPA networks and the other two were specifically addressing comparisons between protected and non-protected areas (Wood and Gardner, 2007; Bell, 2008a; McInerney et al., 2009b) (Table 2.1). These 3 papers were dealing with different geographic areas (Ireland and New Zealand), spatial scales (1 m to 170 Km), taxa (crustaceans and molluscs), and used different molecular markers (microsatellites and randomly amplified polymorphic DNA markers) (Table 2.1).

Species	Geographic region	MPA sampling design	Spatial range Gleick et al.,		LD (days)	Genetic marker	Reference
			Min	Max			
Semibalanus balanoides	Irish Sea, Ireland (Lundy, Skomer MNR and Inishmean, Inishbofin SAC)	MPAs vs. non-MPAs	5	80	28-42 [1]	Microsatellites	Bell, 2008a
Nucella lapillus	Irish Sea, Ireland (Strangford Lough MNR)	MPA vs. non-MPA	5	8	0 [2]	Microsatellites	Bell, 2008a
Nucella lapillus	Irish Sea, Ireland (Strangford Lough MNR)	MPA vs. non-MPA	0.001	170	0 [2]	Microsatellites	McInerney et al., 2009b
Scutellastra kermadecensis	SW Pacific Ocean, New Zealand (Kermadec Islands MR)	MPAs network	0.5	150	4-10 [3]	RAPDs	Wood and Gardner, 2007
Siphonaria raoulensis	SW Pacific Ocean, New Zealand (Kermadec Islands MR)	MPAs network	1	7	4-10 [3]	RAPDs	Wood and Gardner, 2007

Table 2.1. Genetic connectivity studies using invertebrate temperate species for the evaluation of MPA design. LD= Larval dispersal; MR=Marine Reserve; MNR=Marine Natural Reserve; MPA=Marine Protected Areas; RAPDs= Randomly Amplified Polymorphic DNA markers. [1] Larval dispersal based on Dufresne et al., ; [2] No planktonic larvae dispersal; [3] Estimation of the larval dispersal based on *Patella* species Dodd, 1957b

All the species under study were intertidal and differed in reproductive behaviour and pelagic larval duration (PLD). They ranged from species with no larval dispersal Bell, 2008; medium PLD, 4 to 10 days (*Scutellastra kermadecensis, Siphonaria raoulensis (Dodd, 1957*; Wood and Gardner, 2007; Bell, 2008b) and long PLD, 28-42 days (*Semibalanus balanoides*; Dufresne et al., ; Bell, 2008a). All of them used structured sampling design to test the effectiveness of MPAs. McInerney McInerney et al., 2009c evaluated the function of the Strangford Lough Marine Reserve (Ireland) analysing the spatial genetic structuring of the dogwhelk *Nucella lapillus.* They included in the sampling design five populations within the Marine Reserve at least 15 kilometres apart, and two populations' outside the reserve. Similarly, Bell, 2008b analysed connectivity between MPA and non-MPA populations, where the MPA populations were situated on island while the non-MPA were on the mainland. Bell revealed a common pattern for the two species, with lower genetic differentiation found between adjacent sites on the mainland (no MPA) than between the island MPAs suggesting that special consideration must be given to the MPA located on islands, because they may not be well connected with the surrounding populations. Similarly, Wood and Gardner (2007) examined genetic structuring and connectivity among populations of 2 intertidal limpets in populations within the Kermadec Islands Marine Reserve network (Australia) without taking into account populations outside the reserve.

2.4. MPA connectivity and sampling design

The literature survey showed that most papers dealing with effectiveness of MPAs in temperate regions using marine invertebrates as target taxa, do not apply a sampling design structured to test this hypothesis. Mokhtar-Jamai (2011) studied the genetic structure of the red gorgonian *Paramuricea clavata* in the Western Mediterranean, among the regions of Medes, Marseille and North Corsica. Although these regions are considered as protected areas, their experiment was not design to formally test the efficiency of MPAs; but provides

valuable information to understand genetic structuring in this threatened species and might be useful for future MPA studies.

Almany et al., 2009 provided general recommendations for the location, size and spacing of reserves, and considerations for maintaining genetic diversity based on larval dispersal data. However, they did not provide details on the structure of the experimental design. To test the effectiveness of MPA and/or a MPAs network appropriate experimental designs, as well as selection of species and molecular markers are required.

2.4.1. Sampling design

The primary goal to take into account to develop an effective sampling design is to clearly formulate the hypothesis to test. In order to have an efficient MPAs network, each MPA populations has to be supported by larvae that settle within that reserve's boundaries (self-recruitment) to maintain local populations, while at the same time an exchange of larvae between MPAs (source-sink system) should occur to maintain genetic diversity (Berumen et al., 2012). To evaluate the effectiveness of MPAs, connectivity, genetic variability within and between the MPAs has to be compared. The hypothesis should test if populations from protected sites have higher levels of genetic variation compared to populations from unprotected sites, as an expression of more "healthy conditions" Frankham, 2005b, and quantify genetic structuring between MPAs. A comparative study should allow to ascertain: 1) the role of MPA as "source" of larvae to non-protected areas (spills over effect), ensuring the replenishment of depleted or declining populations (demographic connectivity); 2) the relationship between anthropogenic pressures and the genetic variability of populations (evolutionary connectivity); and 3) MPA size required to support viable populations.

A sampling design set up for the study of an MPA, should be replicated in other randomly selected MPAs, to be able to generalise the observed patterns and to avoid confounding effects (Benedetti-Cecchi et al., 2003). A robust comparison requires selecting sites with similar environmental conditions, considering the different extent of MPAs and local geography, excluding unusual/isolated sites to avoid bias while describing connectivity patterns (Hellberg, 1996). In several studies on genetic structuring sample from a variety of different habitats (e.g.

caves, walls, shallow, deep, mainland, island) have been merged in a single analyses, and an ‘a posteriori’ discussion attributed the observed patterns to site features, without considering the lack of a specific hypothesis to test and replication of the considered habitat features. Lack of structure and replication in the sampling design creates major difficulties in the interpretation of the results, facilitating the confounding effect. To avoid this problem a correct replication of each investigated factors is needed.

Demographic structure of populations and environmental variable, such as currents, tides, temperature, and pollution, are important aspects to consider in the sampling design. In fact, combining genetic connectivity patterns with demographic data and knowledge on environmental parameters integrating in a fine-grained biophysical model should allow elucidating the distribution and diversity of populations. Di Franco et al., 2012 combined visual observation, oceanographic models and genetics (microsatellites) in the evaluation of protected and non-protected areas. Sampling area, number of sampling sites, and distance between sampling sites will depend primarily on the MPA size and on the species selected. Recent studies showed a high variation in larval dispersal among marine species, with larvae recruiting within the source population, while others dispersing over large distances (10–100s of km; Jones et al., 2009). Scales of genetic structuring of a species provide tips about the appropriate sampling distances within and outside MPA. In intertidal limpet species, which have high dispersal potential and are ubiquitous, distances between sampling sites could range between 10 and 30 km (Bell, 2008b). In octocoral species, conversely, dispersal capability is limited and they are patchily distributed, therefore distance between sampling sites has to be less than 5 km (Hellberg et al., 2002; Mokhtar-Jamai et al., 2011; Costantini et al., 2013). The number of sampling sites has to be decided depending on the geographic extension of the study area. Moreover, the sampling site has to be delimitated to properly characterise the genetic structure of the population avoiding clones and collection of closely related individuals. For example, in intertidal gastropods (Bell, 2008b) individuals were collected over 8-10 m apart, while in coral species 1-2 m distances are enough (Costantini et al., 2007a).

2.4.2. Species selection

Marine invertebrates show great species diversity and a variety of life-history traits, they have a widespread distribution and high abundance per unit area; therefore they are good model organisms to investigate connectivity patterns. Among them, benthic species better reflect current gene flow pattern, since gene flow is mainly mediated by the early life stages (gametes, zygotes, larvae, juveniles), while adults are fairly stationary. Since patterns in genetic connectivity differ among species (Coleman et al., 2011), a multispecies approach would allow investigating the occurrence of patterns of connectivity common to several species, differing in their biological and ecological features (e.g. Toonen et al., 2011; Berumen et al., 2012).

Identifying discontinuity zones in genetic structure shared by several species would allow an objective validation of biogeographic zone and of geographical evolutionary units. Conversely, lack of common patterns among species may support occurrence of species-specific barriers to larval dispersal, which may be related to biological peculiarities (e.g. life history, reproductive behaviours, larval type, and dispersal) or to the demographic history of the local population. Only in the last two years studies using a multispecies approach were carried out. All of them suggested that this innovative approach allow a better understanding of the evolutionary dynamics of the system as a whole, and therefore the ability to better scale conservation measures (Kelly and Palumbi, 2010; Toonen et al., 2011; Drew and Barber, 2012) in temperate habitats, and (Selkoe and Toonen, 2006) in tropical seas, used a large number of marine species (including cnidarians, gastropods, crustaceans, echinoderms, reef fishes and marine mammals), revealing patterns of genetic structure undetectable in single species. In fact, as Drew and Barber (Drew and Barber, 2012) stressed "selecting a reserve design based on any single species would not adequately represent the evolutionary and ecological dynamics expressed in the other species". Nevertheless, practical constrains (time and costs) do not allow a complete analysis of the genetic patterns of the species occurring in the assemblages, therefore it is important to select species with different ecological and biological features and living in different habitats (e.g. subtidal vs. intertidal).

2.4.3. Molecular markers

The choice of the molecular markers is also an important point to consider. A variety of markers became available in the last decade. The selection of the suitable marker depends on the species of focus and the spatial and temporal scales of interest (see as review Hellberg et al., 2002). The most widely used markers for genetic connectivity studies are microsatellite markers (tandem repeats of 2–10 base pair nucleotide; e.g. Bell, 2008b; McInerney et al., 2009a) that for their high polymorphism provide enough variability to define genetic pattern of connectivity. To date the main problem related to these markers is that they are species specific and only a limited number, if any, is available for most species (for example 5 microsatellites in *Semibalanas balanoides* in Dufresne et al., 1999; Bell, 2012). In case of multispecies analysis this could lead the choice of a more "common" molecular marker such as the mitochondrial DNA. The most common used mitochondrial marker is cytochrome oxidase c subunit 1 Rousset, 2008 gene, since it is one of the most variable regions in the mitochondrial genome (a part for corals and sponges, see Shearer and Coffroth, 2006). The combination of both mitochondrial and nuclear (e.g. ITS, microsatellites) provide the best approach for studying the evolutionary and contemporary gene flow (e.g. Teske et al., 2010), however it is rarely applicable.

2.5. Implementation of the sampling design guidelines

The present study highlighted how little is known on connectivity among MPAs in temperate marine habitats. Guidelines for the implementation of effective sampling designs are needed. The power of multispecies studies and of the use of markers differing in the evolutionary rates has been stressed. To date, only very few studies integrated effective sampling design in the assessment of connectivity among MPA (but see Di Franco et al., 2012 in the Mediterranean Sea and Foster et al., 2012 in the Caribbean). They are important starting points to take in account when a new study as to be set up to evaluate the effectiveness of the already existing marine protected areas.

The Mediterranean Sea contains unique habitats and a variety of endemic species (Bianchi and Morri, 2000), which conservation is among the priorities in

marine sciences. Despite the high number of Mediterranean MPAs, their design and management is not based on a holistic approach and data on connectivity are often lacking. International research projects provide an appropriate framework for the implementation of effective sampling designs, and the Mediterranean could be a suitable study case to assess genetic variability and population structure across MPAs through the whole basin. In each MPA a hierarchical sampling design should be implemented, including replicated sites inside and outside of the MPAs. Distance among sites depends on the MPA size, the geomorphological and environmental characteristics, and the target species. Species with different life history traits and inhabiting different reef zones (e.g. intertidal vs. subtidal, cliffs vs. caves) should be analysed (e.g. molluscs as *Patella* in rocky intertidal, gorgonians as *Eunicella* in subtidal). Different nucleotidic polymorphic markers (both nuclear and mitochondrial) have to be used to assess the extent and patterns of gene flow, as well as the larval dispersal capability. The genetic results together with ecological, demographical, biological and oceanographic data will be essential in planning effective MPA networks and management strategies for the conservation of marine biodiversity.

2.6. Acknowledgements

This research was funded by the European project 'Training Network for Monitoring Mediterranean Marine Protected Areas' (MMMPA: FP7-PEOPLE-2011-ITN) [grant number 290056]. PMP was supported by a 'Marie Curie Training Network for monitoring Mediterranean Marine Protected Areas' (Early stage researcher). FC was supported by a research fellowship (Assegno di Ricerca) of the University of Bologna.

Chapter III: Morphometric and genetic tools for Marine Protected Area monitoring

Marine Protected Area of Portofino. Photo source: Patricia Marti Puig

Publication note: A modified version of the following chapter is in preparation for submission: *Patricia Marti-Puig, Massimo Ponti, Paolo G. Albano, Federica Costantini & Marco Abbiati, Morphological and genetic tools for MPA monitoring in two sympatric limpets (Patella rustica and Patella caerulea) in the Western Mediterranean MPAs (in prep)*

3.1. ABSTRACT

Many marine organisms show a high morphological variability, which often represents a result of the phenotypic plasticity towards different environmental conditions, such as wave exposure, substrate type and habitat. This variability can lead to species misidentification using traditional methods, with serious implications for monitoring activities and application of conservation policies, such as in the Marine Protected Areas. In the present study, distinction and variability in morphology of two common limpets, *Patella rustica* and *P. caerulea*, was studied among Marine Protected Areas in the western Mediterranean Sea using genetics and digital morphometric techniques. Genetic marker COI was used to identify the species. Morphological variability of the two species was analysed using digital morphometric shell characters and shape analysis. Morphometric measures and shape analysis showed high morphological variability in both species, higher in *Patella caerulea*. Our results showed that some morphological traits (mean circularity, ratio height/longest diameter and solidity) and shape descriptors are useful tools to discriminate and identify variability among and within the species. Our results underline the importance of combining the use of genetic and digital morphometric tools for MPA monitoring.

3.2. Introduction

Many marine organisms show a high morphological variability, which often represents a result of the phenotypic plasticity towards different environmental conditions, such as wave exposure, substrate type and habitat (Padilla, 2013). Phenotypic variability is important because it increase resilience of populations as conditions change (Watters et al., 2003). However, this variability can lead to species misidentification with serious implications for monitoring activities, such as in the Marine Protected Areas, and additional methods should be used.

Genetic analyses, when markers are available, can be useful tools to identify species difficult to distinguish morphologically (Hebert et al., 2003). A segment of the mitochondrial gene cytochrome c oxidase subunit I (Rousset, 2008) is the most commonly used barcode region in species identification of many invertebrates (Kress and Erickson, 2008). COI has been already used to discriminate among molluscs species and in particular *Patella* species (Mauro et al., 2003; Sá-Pinto et al., 2005; Fauvelot et al., 2009). However, species identification based on only few morphological characters or on a single molecular marker, have a risk for misidentification which can raise conservation risks (Will et al., 2005; Zachos et al., 2013).

Digital morphometric methods offer a good alternative to traditional methods to select proper characters to identify species. These powerful techniques can capture differences in structures that are not easily observed through traditional types of measurements or by the naked eye. Digital images can be automatically analysed using a computer softare (e.g. ImageJ) in order to measure the size of the organism by a semi-automatic identification of the outline. This method have been successfully used to disentangle the intraspecific variability and phylogeny of a wide range of species, including shell morphology in mollusks (e.g. Kotsakiozi et al., 2013). Additionally, if there is the need to capture much higher proportion of the morphological information, geometric morphometric tools such as analysis based on Fourier Shape Descriptors (EFDs) offer the possibility to analyse and compare morphological shape independently of the size (Kuhl and Giardina, 1982; Crampton, 1995; Van Bocxlaer and Schultheiß, 2010). EFDs can delineate any form of two-dimensional shape with a closed contour and have been previously used in other gastropod species for studying the shell shape (Williams et al., 2012;

Puillandre et al., 2009; de Aranzamendi et al., 2010; Costa et al., 2010; Van Bocxlaer and Schultheiß, 2010; González-Wevar et al., 2011; Ramajo et al., 2013). EFDs offer several important advantages, such as the invariance with respect to scale, rotation and starting position of chain coding contour tracing (Iwata and Ukai, 2002). Moreover, it does not require landmarks and offers the possibility to visual size shape variation that might be difficult to describe (Hiraoka et al., 2004).

Limpets, as those of the genus *Patella*, often show a high phenotypic variability and plasticity related to environmental conditions (Tlig-Zouari et al., 2011). In some Atlantic and Mediterranean localities, it is known that species belonging to *Patella* genus exhibit such a wide variability in shell coloration and morphology that even an experienced observer can be confuse (Moore, 1934; Evans, 1953; Bacci and Sella, 1970). *Patella caerulea* L. 1758 and *Patella rustica* L. 1758 are the most common Mediterranean species of the genus. They usually occur sympatrically in different zone on the Mediterranean rocky shores (Bannister, 1975). Genetic analyses along with morphometric methods may allow to disentangle between morphologically similar species and to evaluate the morphological plasticity in relation to the environmental variability.

The aim of this research was to evaluate the distinction and variability of two sympatric limpets (*Patella rustica* Linnaeus 1758 and *Patella caerulea* Linnaeus 1758) in four western Mediterranean MPAs using different morphometric and genetic tools, which can be applied for MPA monitoring in the field.

3.2. Material & Method

3.2.1. Field sampling

Limpet specimens, possibly belonging to *Patella rustica* and *P. caerulea*, were collected in 2 sites along 4 MPAs in the western Mediterranean coast: Cabo de Palos (Spain), Port-Cros (France), Portofino and Tavolara (Italy). At each site at least 30 individuals for each putative species were carefully collected, avoiding breaking the shells, within an area of 100 m^2 in the rocky intertidal habitat. Samples were preserved in 90% ethanol and maintained at 4 °C until processing. After samples had been taken for DNA extraction, each shell was cleaned by removing the remaining tissue and properly labelled for morphometric analysis.

3.2.2. Species identification by molecular markers

About 2 g of tissue from the foot of the mollusc was used for DNA extraction using the protocol REDExtract-N-Amp kit (Sigma–Aldrich). Amplification of a portion of 430 bp of the mitochondrial COI was carried out with universal primers (Folmer et al., 1994) in a final volume of 25 µl consisting of 4 µl of $MgCl_2$ 25 mM, 5 µl of buffer 10X, 0.5 µl dNTP 10 mM, 0.5 µl of each primer 10 µmol, 0.2 of Taq polymerase (Promega) and 2.5 µl of template DNA. Polymerase chain reaction (PCR) was performed in a thermal cycler (SimpliAmp™ Thermal Cycler) as follows: 94 °C for 3 min, 25 cycles at 94 °C 45 min, annealing at 48 °C for 1.5 min, extension at 72 °C for 2 min and with final elongation at 72 °C for 5 min. PCR products were purified and sequenced by Macrogen Inc. Sequences were checked manually, aligned and edited using the software Geneious Pro 5.3. Sequences were blasted in genbank.

A phylogenetic tree was produced with the 203 COI sequences with the software Geneoius pro using Bayesian inference as implemented in MrBayes (Huelsenbeck and Ronquist, 2001). Substitution Model used was HKY85 with a Chain length of 1.100,000, burn-in Length 100,000 and subsampling frequency of 200. Three COI sequences of *Patella aspera* (Röding, 1798) was used as an out-group (EF462968)

3.2.3. Morphometric shell characters

Only shells that were in good shape (not broken, n = 200) were used for morphometric analyses. Shell height (*H*) was measured using a digital calliper with a precision of 0.001 mm. Digital images of the shells were acquired on a white background. Photographs of the specimens were taken with an Olympus SP-350 camera using the following settings: metering in manual (1/30 F 8.0), Focus: S-macro, Zoom in max wide-angle, ISO: 50, with an image size of 3264 × 2448 pixels.

Shell shape and size parameters were calculated by digitalizing the outline using the software ImageJ (http://imagej.nih.gov/ij/). Measured and calculated shape descriptors were: Area (*A*), Major axis and Minor axis of the fitting elipse, Maximum Diameter or Feret's diameter (D_{max}), Aspect ratio (*AR* = Major

Axis/Minor Axis), Roundness (*ROUND*) which is calculated with the formula 4 x (A/πi x Major Axis^2) and Solidity (*SOL*) which is a measure of how “ruffled” the borders are (Ar/Convex area) (Figure 1). Height/Area (*H/A*), Height/Maximum Diameter (*H/MD$_{max}$*) were also calculated to know the proportion which is independent of the size of the shell

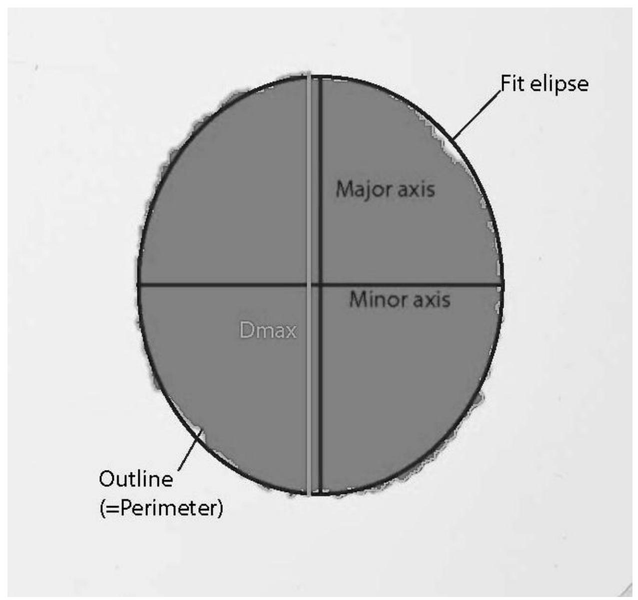


Figure 3.1. Example of some descriptors calculated with image J software.

The morphometric measures relatively of the size of the shells (Area and Major) were compared among protected and non-protected sites. An ANOVA was performed comparing Area and Major of each species respect to the protection using the software R. Factor Protection (nested in Site and MPA) were analysed for each variable. Additional morphometric measures (*AR*, *H/A*, *H/MD$_{max}$*, *ROUND* and *SOL*) were compared between species, among sites within locations for each species. Differences in mean values were tested with an univariate permutational analysis of variance (PERMANOVA; Anderson and Robinson, 2001, Anderson and Braak, 2003) based on Euclidean distance of untransformed data. Factors Species (Fixed), Location (Random) and site (nested in Location, Random) were analysed for each variable. Significant biometric measurements between species were analysed by Principal Component Analysis (PCA) with the software R (Venables and Smith, 2005).

3.2.4. Elliptic Fourier Descriptors (EFDs) method

The same digital images as for morphometric measures were used for the EFD approach. Shells were always positioned in the same orientation. Photos were edited in Adobe Photoshop and then converted to MS Windows bitmap. A series of outline shape analyses were performed using the SHAPE software package (v. 1.3) (Iwata and Ukai, 2002). Digital images were binary-encoded to produce a closed curve. Histogram and Ero Dil Filter were manually adjusted in order to adequately capture the contour of the shell. A chain-code of contour of each shell was created automatically using SHAPE-ChainCoder (Freeman, 1974). The coefficients of EFDs were calculated by discrete Fourier transformation from chain-code (Kuhl and Giardina, 1982) using SHAPE-Chc2Nef. Fourier reconstructions using increasing numbers of harmonics, compared to the original outline, was used to estimate that 10 harmonics were sufficient to reconstruct the outlines with high accuracy. EFDs were mathematically normalized to be invariant with respect to scale, rotation or location and starting position of chain coding contour tracing in accordance with the procedures suggested by Kuhl and Giardina, 1982. Normalization was based on the longest radius and outlines were aligned in Chc2Nef. A principal component analysis of the variance-covariance matrix from the EFDs coefficients was performed using SHAPE-PrinComp to summarize the information contained in the normalized EFD coefficients (Rohlf and Archie, 1984). The shape variation accounted for by each principal component was visualized using SHAPE-PrinPrint using the method of Furuta et al., 1995. The first 5 PCA scores (which explained at least 5% of the variability) were used as shape descriptors in multivariate analysis of variance (MANOVA) and discriminant function analysis (DFA) to assess for shape differences between species. The variation explained by the first two principal components was plotted using an R routine (Venables and Smith, 2005).

Shape descriptors were analysed with a multivariate analysis of variance (MANOVA) using the first 5 PCA scores from the EFD results. MANOVA was performed with R with the package vegan with Random Forrest distance with 10000 permutations (Venables and Smith,). A linear model was calculated using the PC scores in respect to the species per site (nested in location).

3.3. Results

3.4.1. Phylogenetics and species identification

A phylogenetic tree of the individuals is shown in Figure 3.2. The phylogenetic analysis showed that the individuals collected belonged to three genetic clusters with 100 of bootstrap probability. All genetic sequences were blasted in Genbank and the three genetic groups were identifed as *Patella caerulea* (n=99), *Patella rustica* (n=101) and *Patella aspera* (n=3) with an accurancy of more than 99%. There were not relationship found between genetic sub-clusters and geographic location of the species.

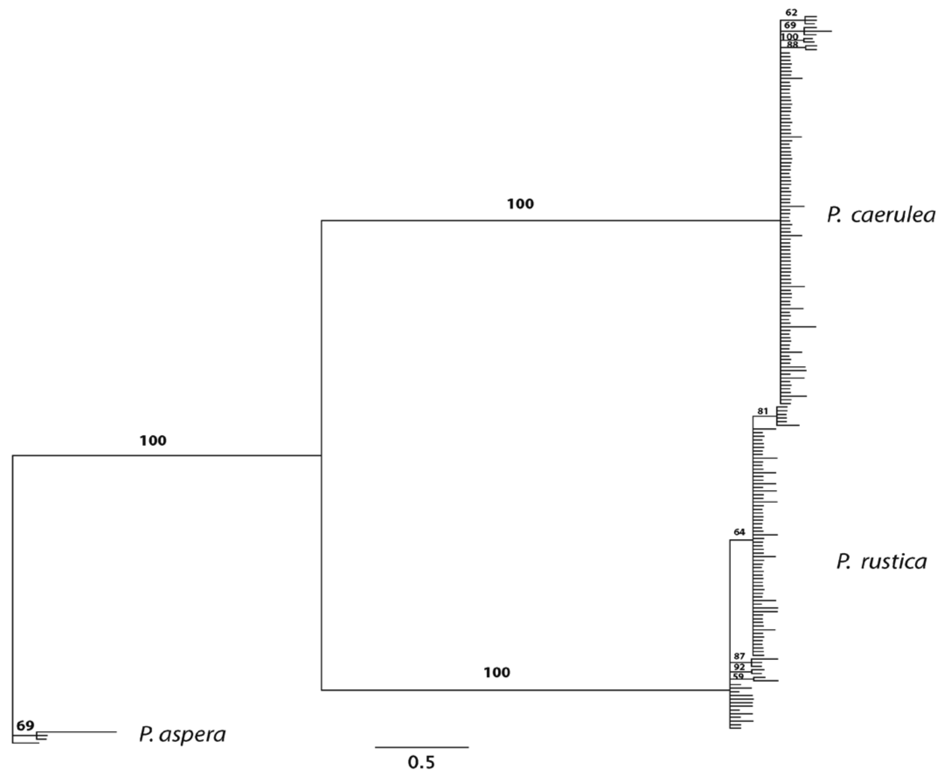


Figure 3.2. Bayesian phylogenetic tree of 200 individuals of *P. caerulea* and *P. rustica*. The species *P. aspera* is shown as an outgroup. Numbers correspond to the bootstrap values.

3.4.2. Morphometric distinction

The result of the PERMANOVA analyses using the morphometric measures is represented in Table 3.1. The parameters H/MD_{max} and Solidity are the best to discriminate both species. Solidity and *H/A* are able to discriminate the species within sites.

A PCA with the significant shape descriptors (*H/A*, H/MD_{max} and *SOL*) is represented in Figure 3.3. The PCA also showed that H/A, H/MD and SOL were good shape descriptors to discriminate the species. Average measurements of H/MD and Solidity per sites are represented in S3.1. Solidity and H/A are higher in *P. rustica* respect to *P. caerulea* (S3.1).

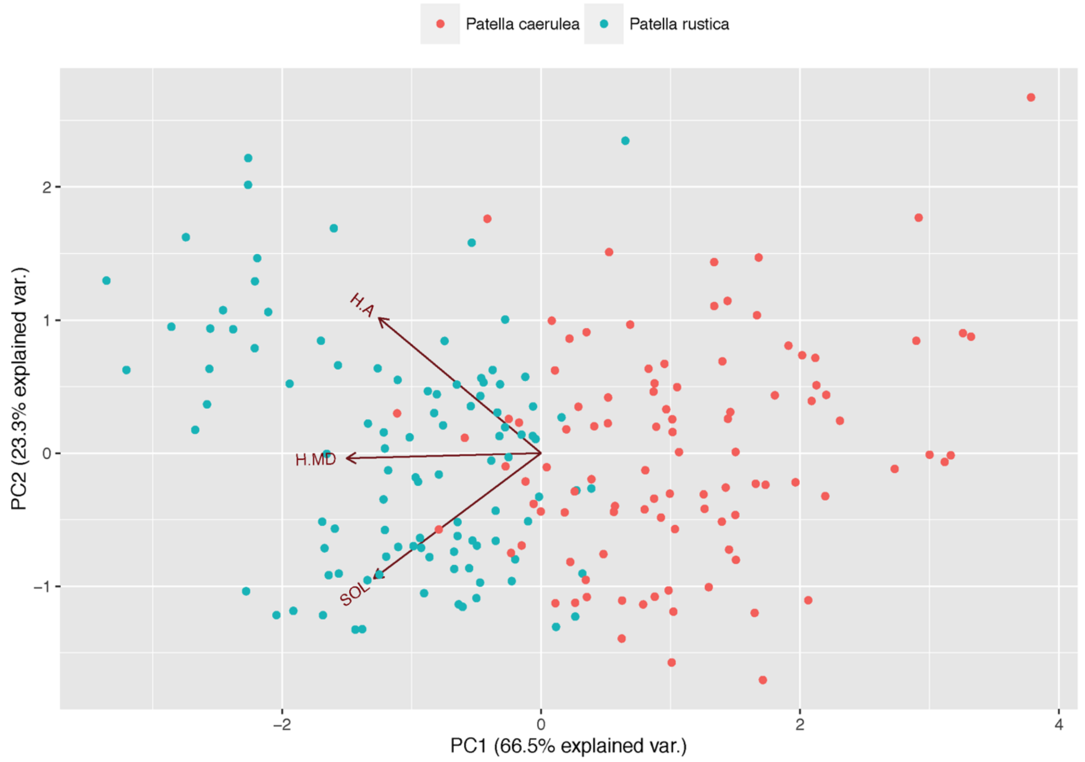


Figure 3.3: PCA with the significant shape descriptors (*H/A*, H/MD_{max} and *SOL*).

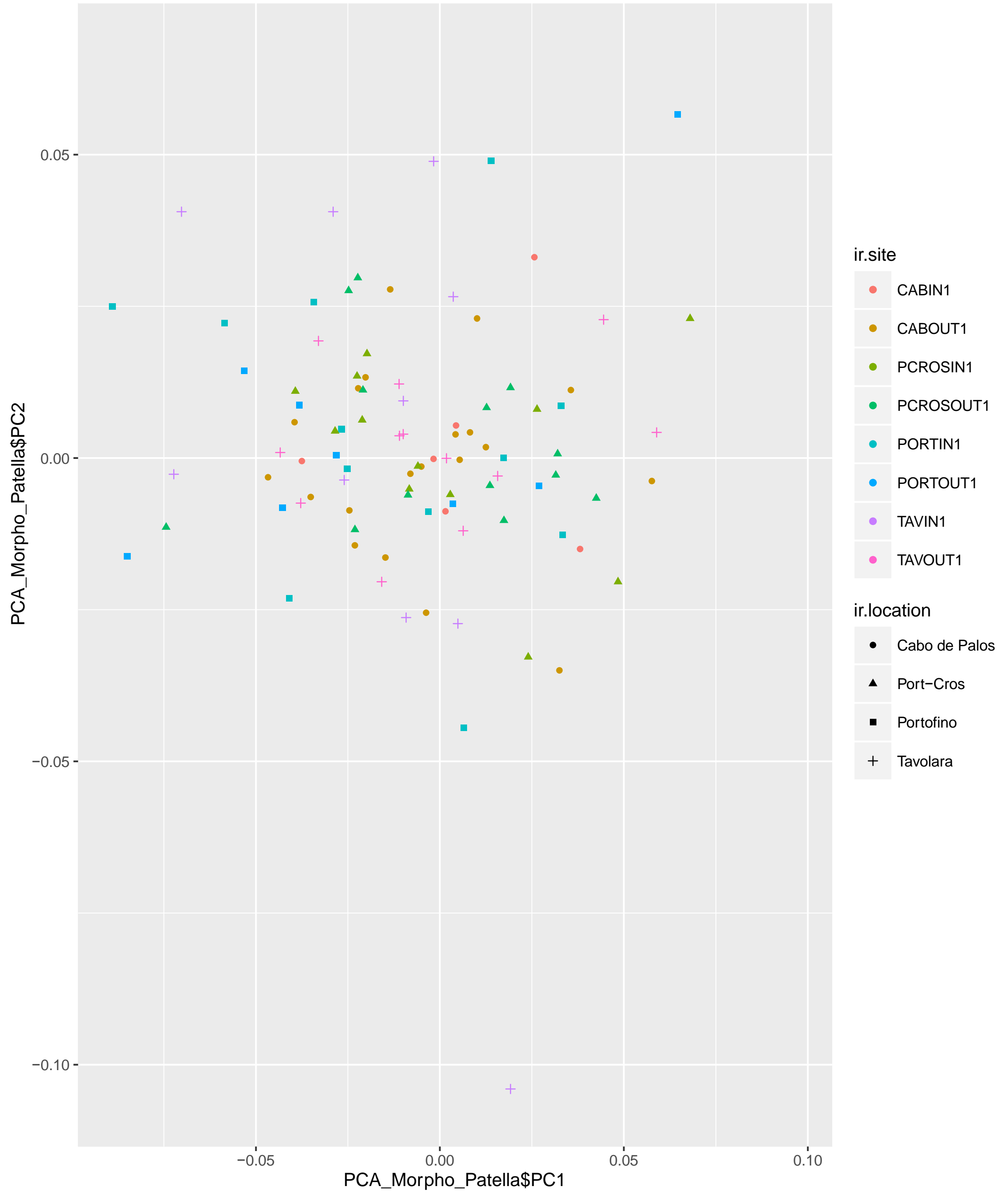
	Species				***Sp x Lo***				***Sp x Si (Lo)***				***Res***
	MS	*Pseudo-F*	*P(perm)*		*MS*	*Pseudo-F*	*P(perm)*		*MS*	*Pseudo-F*	*P(perm)*		*MS*
AR	*0.004*	*1.132*	*0.357*	*ns*	*0.004*	*2.146*	*0.238*	*ns*	*0.002*	*0.607*	*0.662*	*ns*	*0.003*
H/A	*0.003*	*9.490*	*0.061*	*ns*	*0.000*	*1.169*	*0.424*	*ns*	*0.000*	*8.758*	*0.000*	***	*0.000*
H/MD	*0.339*	*40.739*	*0.021*	***	*0.008*	*6.400*	*0.055*	*	*0.001*	*1.517*	*0.198*	*ns*	*0.001*
ROUND	*0.001*	*0.622*	*0.468*	*ns*	*0.002*	*2.910*	*0.167*	*ns*	*0.001*	*0.607*	*0.655*	*ns*	*0.001*
SOL	*0.009*	*12.405*	*0.059*	*	*0.001*	*2.452*	*0.199*	*ns*	*0.000*	*3.183*	*0.015*	*	*0.000*

Table 3.1.Summary of PERMANOVA analyses using the morphometric measures of Aspect Ratio (*AR*), Roundness (*ROUND*), Height/Area (*H/A*), Height/Maximum Diameter (H/D_{max}) and Solidity (*SOL*). *** high significance ($p<0.0001$), **medium significance ($p<0.001$), *low significance ($p<0.01$). Ns: not significant.

	Df	Sum Sqs	Mean Sqs	F.Model	R2	Pr(>F)	
Species	1	2.344	2.344	6.140	0.032	1.00E-04	***
Site:Location	7	3.567	0.501	1.335	0.049	0.0012	**
Species:Site:Location	7	3.092	0.442	1.157	0.043	0.05779	.
Residuals	167	63.768	0.382	0.876			
Total	182	72.772	1				

Table 3.2. MANOVA analysis using the EFDs. *** high significance (p<0.0001), **medium significance (p<0.001), *low significance (p<0.01). Ns: not significant.

EFD using the first 5 PCs scores were able to discriminate the species, also within sites (Table 3.2). The first 5 components explained the 69.9% of variability. Higher morphological variability was present for *P. caerulea* (Figure 3.4), which was not related to the location or site. *Patella caerulea* showed different morphotypes, some more similar to *P. rustica* ("oval shape") and other extreme morphotypes that were clearly distinct showing a "star shape" (S3.3).



3.4. The PCA for the species *Patella caerulea* using EFDs.

3.5. Discussion

In this study genetics with morphology were studied in order to discriminate and identify the morphological variability of the two species *P. caerulea* and *P. rustica*. Both genetic and morphometric methods were useful to distinguish the species. The morphometric measures *H/A*, H/D_{max} and *SOL* were significantly different between species, which means that *P. rustica* can be distinguished by *P. caerulea* because it is usually more round and has a higher height in respect to the area or the maximum diameter. A previous study carried out along the Sicily coasts on the genetic and morphological differences among *Patella rustica*, *Patella caerulea* and *Patella aspera* was clearly able to distinguish genetically between species but discriminant analysis of simple morphometric shell characters (shell length, shell width and shell height) failed to discriminate the species morphologically (Mauro et al., 2003). This is easily understandable by considering that the measures used in this study were not morphometric ratios but were absolute measures that depend on the age and growth rate of individuals. In the present study morphometric shell measures independent by the whole shell size were used.

EFDs descriptors were also able to discriminate the species, showing a higher variability in shell shape in *P. caerulea.* Williams et al. (2012) was also able to distinguish between two closely related species of the gastropod *Lunella* using EFDs and genetic methods. EFDs were also useful to identify the morphological variability and shape types of *P. caerulea.* These morphological variability could be explained by the environmental heterogeneity of the habitat (Watters et al., 2003).

3.6. Conclusion

Despite the morphological differences among species appeared consistent, a large local morphological variability was found. These results underline the importance of the use of morphometric and shape analysis approaches when taxonomic identification is challenging and to identify and quantify morphological

variability. These tools have the potential to be used in the field for future MPA monitoring, were often the identification must be done without specimens withdrawal and when there is the need to quantify variations size and shape of the individuals. Further understanding of the importance of morphological and genetic variability on adaptability and species resilience could help to guide future conservation strategies.

3.7. Acknowledgments

Sampling was done in accordance with national laws, and authorisations were granted by local MPAs authorities. This study did not involve endangered or protected species. This research was funded by the European project 'Training Network for Monitoring Mediterranean Marine Protected Areas' (MMMPA: FP7-PEOPLE-2011-ITN; grant number 290056). Involved MPAs were associated partner of the MMMPA projects. PMP was supported by the MMPA project, as early stage researcher, and this study is part of her PhD. AV was supported by a research fellowship (Assegno di Ricerca) of the University of Bologna and 2010-11 PRIN project prot. 2010Z8HJ5M -Coastal bioconstructions: structures, functions, and management. Thanks to Augusto Navone, Jose Antonio García-Chartón, Ramón García, José Pereguiñez, Marco Palma and Ubaldo Pantaleo for their assistance during the sampling.

3.8. Supplementary materials

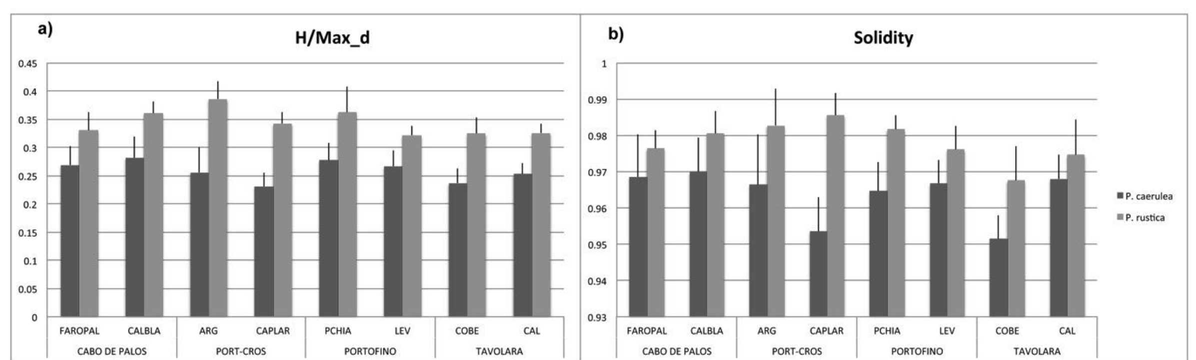
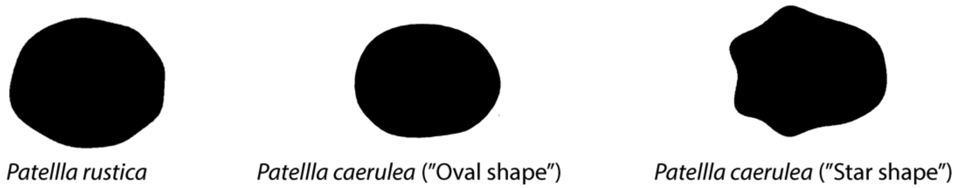


Figure S3.1. H/Dmax (a) and Solidity mean (b). FAROPAL=Faro Cabo de Palos, CALBLA=Calblanque, PCHIA=Punta Chiappa, LEV=Levante, COBE= Corallina Beach, CAL=La calleta).

	Eigenvalue	Proportion(%)	Cumulative(%)	> 1/37
Prin1	1.20E-03	29.8986	29.8986	*
Prin2	5.51E-04	13.7734	43.6721	*
Prin3	4.63E-04	11.5849	55.257	*
Prin4	3.09E-04	7.7317	62.9887	*
Prin5	2.76E-04	6.8979	69.8867	*
Prin6	1.81E-04	4.52	74.4067	*
Prin7	1.46E-04	3.6408	78.0475	*
Prin8	1.27E-04	3.1878	81.2353	*
Prin9	1.14E-04	2.847	84.0823	*

S3.2. Eigen values of EFDs and their proportion



S3.3. Examples of shapes generated with EFDs

CHAPTER IV: GENETIC DIVERSITY AND CONNECTIVITY FOR THE EVALUATION OF MARINE PROTECTED AREAS

Cabo de Palos Marine Protected Area. Photo source: Patricia Marti-Puig

Publication note: A modified version of the following chapter is in already submitted in the journal Marine Environmental Research: *Patricia Marti-Puig, Federica Costantini, Massimo Ponti, Adriana Villamor, Marco Abbiati. "Genetic diversity and connectivity in two intertidal limpets across Marine Protected Areas in north-western Mediterranean". Marine Environmental Research (submitted)*

4.1. Abstract

Marine Protected Areas (MPAs) are intended to protect species diversity and ensure persistence of species. For achieving this purpose, MPAs should be effective in terms of maintaining genetic diversity and connectivity at different spatial-temporal scales. Genetic variability and population connectivity of two widely distributed limpets (*Patella caerulea* and *P. rustica*) were analysed inside and outside four MPAs in the western Mediterranean Sea using mitochondrial and microsatellite markers. No effect of protection on genetic variability was observed in either species. The mitochondrial marker revealed limited genetic structure among MPAs in the north-western Mediterranean for both species. Within each location, different patterns of genetic structure and connectivity were observed depending on the species and local hydrodynamic features. The genetic monitoring presented, provided estimates of connectivity useful to assess MPAs effectiveness and should be included into the monitoring and spatial management plans of MPAs.

Keywords: genetics; gene flow; dispersion; life history; spatial scale; Marine Protected Areas; management; Mollusca; western Mediterranean Sea

4.2. Introduction

Marine Protected Areas (MPAs) are conservation zones that aim to preserve the environment and maintain species diversity, which are threatened by overexploitation, pollution and other human disturbance sources (Salm et al., 2000). A well-designed MPA network should, ideally, ensure to maintain population genetic diversity within MPAs (Frankham, 2005a; Bouzat, 2010) and enhance connectivity (by gametes, larvae or adult dispersal) within protected areas, between them and in adjacent habitats (Crowder et al., 2000; Miller and Ayre, 2008; Jones et al., 2009; Planes et al., 2009). Connectivity and genetic diversity, promoted by exchange of individuals between populations, warrant resilience from disturbance and allow to buffer species against risks of local extinctions (Almany et al., 2009). Following disturbance, survivor populations might show reduced viability (through genetic drift and inbreeding), reducing their genetic diversity, their evolutionary potential and their local larval output with consequences for their productivity, growth, stability and interaction with surrounding populations. In this scenario, connectivity with other populations, as larval inputs, can facilitate their recovery. Therefore, understanding diversity and connectivity patterns together with the identification of potential source and sink populations are important aspects to determine the optimal locations and spacing between MPA, and to develop adequate and specific monitoring and action plans (Crowder et al., 2000; Sala et al., 2002).

Despite the protection of the habitats is at the centre of recent international directives (e.g. the European Marine Strategy Framework Directive, 2008/56/EC), too often the MPAs' conservation policies are exclusively addressed to protect commercial species or those listed in international biodiversity conventions, in the hope that this is enough to preserve the entire habitat (Claudet et al., 2008; Claudet et al., 2010; Gaines et al., 2010). However, attention should be also addressed to those species that, although not explicitly protected, play a key role in structuring local assemblages, as many intertidal invertebrates. Few studies have attempted to determine whether MPAs are able to conserve genetic diversity of benthic intertidal species and whether they are effective in promoting connectivity (e.g. Bell, 2008a; McInerney et al., 2009a; Munguía-Vega et al.,

2015). Those studies demonstrate that marine reserves located around islands have limited connectivity compared to mainland (Bell, 2008a), that mesoscale topographic and hydrographic features drive patterns of intra-specific genetic diversity (McInerney et al., 2009b), and that management decisions may be capable of increasing or decreasing genetic diversity of exploited species over relatively short time scales (Munguía-Vega et al., 2015).

Among intertidal invertebrates, limpets deserve particular attention, being grazers able to control rocky shore communities (e.g. Menconi et al., 1999; Benedetti-Cecchi, 2001; Underwood, 2000; Coleman et al., 2006; Burgos-Rubio et al., 2015). These species are potentially threatened by overharvesting both as local delicacy and as bait for fishing, but also by trampling, coastal destruction and pollution (Keough and Quinn, 1998; Guerra-Garcıa et al., 2004; Pinn and Rodgers, 2005; Fenberg and Roy, 2012). The decline of their populations can lead to a wide range of trophic cascade effects (Bosman et al., 1987).

In the Mediterranean Sea *Patella caerulea* Linnaeus, 1758 and *Patella rustica* Linnaeus, 1758 are the most common intertidal rocky shores limpets. These species can be found at different densities, which may depend on environmental factors such as wave exposure, tidal level, type of substrate and topography but also on natural and anthropic pressures (Davenport and Davenport, 2006). They play a key ecological role in the intertidal habitat, grazing on rocky shores and allowing the control of algal grow and influencing the abundance of other animals species (e.g. barnacles; Arrontes et al., 2004).

Previous studies using mitochondrial and microsatellite markers provided some background information on the genetic structure of *P. caerulea* and *P. rustica*, indicating a high genetic diversity and significant differentiation between western and eastern Mediterranean Sea in both species (Sá- Pinto et al., 2010; Villamor A, 2014).

In the present study, the levels of genetic diversity and structuring of *P. caerulea* and *P. rustica* among and within four MPAs in the western Mediterranean Sea has been investigated using a multifactorial hierarchical sampling design. For this purpose, limpet populations were sampled in replicated sites inside and outside MPAs and analysed using mitochondrial and nuclear markers. This work aimed to answer specific questions: 1) are there significant differences in genetic

variability inside and outside MPAs?; 2) is there a significant genetic structuring among populations across MPAs and within them?; 3) are the genetic features of the two species comparable?

4.3. Materials and methods

4.3.1 Sampling design

The genetic structure of *Patella caerulea* and *P. rustica* within and across Marine Protected Areas (MPAs) have been analysed using a multi-scale sampling design. Four locations where MPAs have been established were randomly selected in the western Mediterranean Sea (Figure 1): Cabo de Palos (Spain), Port Cros (France), Tavolara (Italy) and Portofino (Italy). Studied MPAs differed in date of establishment and size (Table 4.1).

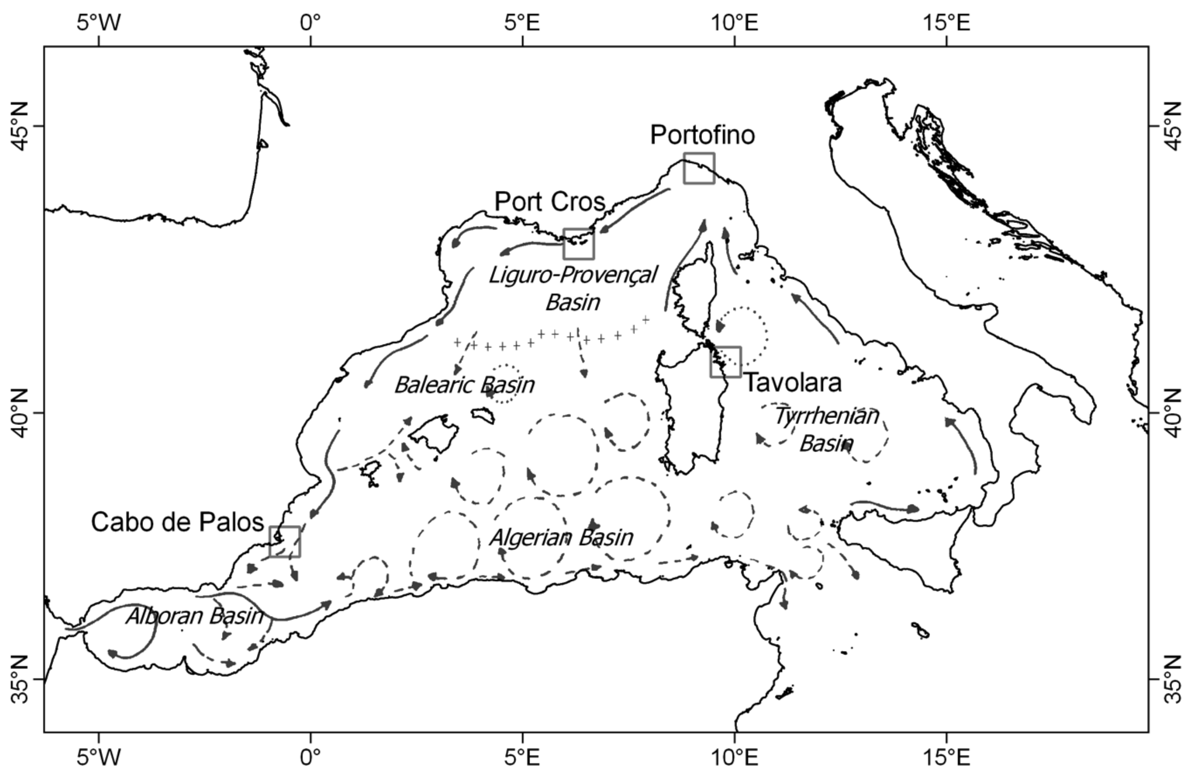


Figure 4.1. Study locations in the western Mediterranean Sea. Grey arrows represent the mean surface circulation (Modified Atlantic Water) according to Millot (1999)): continue lines: more or less steady paths; dashed lines: mesoscale currents throughout the year; dotted lines: wind-induced mesoscale eddies; cross markers: the North Balearic Front (basins names according to López García et al. (1994)); map in Mercator projection, datum WGS 84).

In each location samples were collected in two sites inside (IN) and two sites outside (OUT) the MPA (Table 4.1). Sites were randomly selected about 20 km apart from each other (McInerney et al., 2009b). In each site, up to 30 specimens of *Patella caerulea* and of *P. rustica* were collected within an area of approximately 100 m^2 in the intertidal habitat. *P. caerulea* was more abundant on sheltered lower intertidal shores, while *P. rustica* was mainly found on exposed rocky shores in the upper intertidal. Collected samples were preserved in 90% ethanol and maintained at 4 °C until processing.

Two sites per location (one IN and one OUT of the MPA, labelled 1 in Table 4.1) were analysed using the mitochondrial COI marker to assess regional patterns of genetic structure. All the four sites per location were analysed by microsatellite nuclear markers.

			P. caerulea				*P. rustica*			
Location	**Protection**	**Site code**	**n**	***H***	H_d **± se**	π_d **± se**	**n**	***H***	H_d **± se**	π_d **± se**
Cabo de Palos	protected	CABIN1	22	9	0.658 ± 0.024	0.040 ± 0.006	20	6	0.579 ± 0.028	0.014 ± 0.003
	unprotected	CABOUT1	23	3	0.423 ± 0.022	0.015 ± 0.003	24	11	0.670 ± 0.023	0.024 ± 0.004
Port Cros	protected	PCROSIN1	26	11	0.825 ± 0.012	0.048 ± 0.007	19	11	0.837 ± 0.018	0.033 ± 0.005
	unprotected	PCROSOUT1	23	7	0.783 ± 0.013	0.038 ± 0.006	23	13	0.846 ± 0.015	0.033 ± 0.005
Portofino	protected	PORTIN1	21	9	0.724 ± 0.022	0.045 ± 0.007	23	6	0.717 ± 0.016	0.021 ± 0.003
	unprotected	PORTOUT1	19	6	0.655 ± 0.026	0.032 ± 0.006	24	11	0.714 ± 0.021	0.023 ± 0.004
Tavolara	protected	TAVIN1	24	9	0.659 ± 0.022	0.030 ± 0.005	23	12	0.779 ± 0.019	0.032 ± 0.005
	unprotected	TAVOUT1	24	11	0.841 ± 0.013	0.052 ± 0.007	24	9	0.772 ± 0.016	0.026 ± 0.004

Table 4.1. Study locations and protected and unprotected sampling sites (i.e. inside and outside MPAs)

4.3.2 DNA extraction and markers amplification

About 2 g of tissue from the foot of the limpet were used for DNA extraction using the protocol REDExtract-N-Amp kit (Sigma–Aldrich).

Amplification of 430 bp of the mitochondrial COI was carried out with universal primers (Folmer et al., 1994) in a final volume of 25 µl consisting of 4 µl of MgCl2 25 mM, 5 µl of buffer 10X, 0.5 µl dNTP 10 mM, 0.5 µl of each primer 10 µmol, 0.2 µl of Taq polymerase (Promega) and about 30 ng of template DNA. Polymerase chain reaction (PCR) was done in a thermal cycler (SimpliAmp™ Thermal Cycler) as follows: 94 °C for 3 min, 25 cycles at 94 °C 45 sec, annealing at 48 °C for 1.5 min, extension at 72 °C for 2 min and with final elongation at 72 °C for 5 min. PCR products were purified and sequenced by Macrogen Inc. Sequences were checked manually, aligned and edited using the software Geneious Pro 5.3.

Six microsatellite loci were used to analyse *P. caerulea*: four species-specific loci (Pc11, Pc36, Pc73, Pc38; Fauvelot et al. (2012)), and two loci developed for *P. rustica* (Pru5 and Pru15; Pérez et al. (2008)). *P. rustica* was analysed using 5 microsatellite loci: three species-specific loci (Pru5, Pru15, and Pru8; Pérez et al. (2008)), and two loci developed for *P. caerulea* (Pc11 and Pc38; Fauvelot et al. (2012)). Multiplexed amplifications were done on *P. caerulea* with the loci Pc11, Pc36, Pc73, Pc38, and on *P. rustica* with loci Pc11, Pru5 and Pru15, using the protocols described by the manufacturer (Qiagen Multiplex PCR). Single locus amplifications were done in a volume of 25 µl, consisting of 4 µl of buffer 5x, 1.5 µl of $MgCl_2$ 25 mM, 0.5 µl of each forward and reverse primers 10 mM, 0.5 µl of dNTPS 10 mM, one unit of Taq and 2.5 µl of template DNA 1:40 (30 ng template DNA). The protocol was: 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 40 s at a locus specific annealing temperature (Pérez et al., 2008; Fauvelot et al., 2012), 1 min at 72 °C and a final elongation of 72 °C for 10 min. PCR products were purified and genotyped by Macrogen Inc. Allele sizing was done with the software Peak Scanner (Life Technologies).

4.3.3 Mitochondrial genetic diversity

For each species and site, number of haplotypes (*H*), haplotype (H_d) and nucleotide (π_d) diversity were calculated using the software DnaSP v. 4.50.3 (Rozas et al., 2003). Differences in mean haplotype and nucleotide diversity among locations (4 random levels, two sites each) and between IN and OUT MPA (2 fixed levels: IN and OUT; four sites each) were tested by two-way analysis of variance (ANOVA, $\alpha = 0.05$; Philippart et al., 2011, Underwood, 1996). Cochran's C test was used to check the homogeneity of variances and data transformations were not required. These tests were done using the software GMAV-5 for Windows.

4.3.4 Microsatellite genetic diversity

Observed heterozygosity (H_o), expected heterozygosity (H_e) and inbreeding coefficient Gleick et al., were calculated per site across all microsatellite loci with GENETIX software package v. 4.05 (Belkhir et al., 2004). Rarefied allele richness (*Ar*) and private allelic richness (*pAr*) were calculated after controlling for differences in sample size, using a rarefaction approach implemented in the software HP-RARE 1.1, Kalinowski, 2005. The software GENEPOP (web version http://genepop.curtin.edu.au/; Raymond and Rousset, 1995; Rousset, 2008) was used to test for departures from Hardy-Weinberg equilibrium Schleuning et al., and linkage disequilibrium (LD). The presence of null alleles was examined by estimating null allele frequencies for each locus and sample following the Expectation Maximization (EM) algorithm of Dempster et al., using FREENA (Chapuis and Estoup, 2007).

Effects of locations, IN vs. OUT MPA and sites on genetic diversity indices (*Ar*, *pAr*, H_e and F_{IS}) were tested by a three-way mixed ANOVA using the software GMAV-5 for Windows Folmer et al., 1994; Underwood, 1996). Homogeneity of variances was tested using Cochran's C test, and only *pAr* values have been transformed as square root.

4.3.5 Genetic differentiation and structure

Relationships between the mitochondrial haplotypes were visualized in unrooted haplotype networks, calculated by median joining algorithm with the software NETWORK v. 4.6.1.1 (Bandelt et al., 1999). Loops were solved using the criteria of abundance, origin and least changes.

For the COI data sets, genetic differentiation among sites were estimated with Wright's fixation index Kotsakiozi et al., as implemented in the software ARLEQUIN v. 3.1 (Excoffier et al., 1992) with 10,000 permutations.

Regarding to the microsatellite data sets genetic divergences among all sites were estimated using the F_{ST} estimates of Weir (1996)). Since null alleles were found (see "Results") F_{ST} estimates were also calculated following the ENA method described in Chapuis and Estoup, . The significance of pairwise genotyping differentiation between populations was tested using Fisher's exact tests based on Markov chain procedures in GENEPOP v.3.4 (Raymond and Rousset, 1995). *P*-values were corrected following the False Discovering Rate (FDR) correction for multiple comparisons (Benjamini and Hochberg, 1995). Moreover, to overcome some of the shortcomings of conventional F_{ST} statistic (e.g. F_{ST} tends to underestimate differentiation when heterozygosity is high; Jost, 2008) the pairwise population differentiation were also performed using the D estimator (D_{est}; Jost, 2008) computed with the programme GENODIVE 2.0b27 (Meirmans, 2014). Overall estimates of D_{est}, were calculated from individual loci using a harmonic mean approximation. A Mantel test was performed to compare pairwise-population matrices (F_{ST} *vs.* D_{est}) with 10,000 permutations, using the statistical software R (R Core Team, 2014).

An analysis of molecular variance (AMOVA; Excoffier et al., 1992) implemented in ARLEQUIN was conducted to examine the partition of the genetic variance among locations. Then, within each location, AMOVAs were performed grouping sites IN and OUT MPA.

Occurrence of isolation by distance patterns were tested with a Mantel test (Mantel, 1967) (testing the correlation between geographical distances and the pairwise F_{ST} matrixes) with 10,000 permutations, using the statistical software R (R Core Team, 2014; packages: "adegenet", "pegas", "ecodist). Geographical

distance was estimated using an R script that calculates the shortest distance between two points taking into account the coastline (packages: “raster”, “gdistance”, “rgdal”, "poppr").

At north-western Mediterranean scale and for each location an assignment test was done for the microsatellite data to provide likelihood values to the possible numbers of homogeneous genetic clusters (K) (software STRUCTURE v. 2.3.3; Pritchard et al., 2000; Falush et al., 2003). Simulations included 50,000 generations and 100,000 Markov Chain Monte Carlo (MCMC) steps under the admixture model (Falush et al., 2003), with 20 iterations for each K value. Because sampling location information set as prior information can assist clustering for data sets with weak structure (Hubisz et al., 2009), the LOCPRIOR option was used.

The most likely number of genetic clusters was determined following the Evanno’s procedure (Evanno et al., 2005) as implemented in the web-based software Structure Harvester (Earl and BM, 2012) and checking for the lowest standard deviation of the mean.

STRUCTURE plots were created with CLUMPAK (Kopelman et al., 2015), which compares all runs at each value of K to identify optimal clustering scenarios and uses DISTRUCT (Rosenberg, 2004) to create resulting figures.

For each STRUCTURE analysis, two independent simulations with different starting seeds were performed for each assignment test to assess convergence of the results

The amount and direction of gene flow over the last several generations among sites within locations was estimated by performing a Bayesian-based analysis. MIGRATE-n v.3.6 was used to estimate theta (Θ) and the mutation scaled migration rate M based on microsatellite data (Beerli and Felsenstein, 2001). Theta is defined as $4N_e\mu$ for a diploid system with nuclear microsatellite loci, where Ne is the effective population size and μ is the mutation rate per generation and site. M is defined as m/μ, representing the relative contribution of immigration and mutation to the variability brought into the population, whereas m is the fraction of new immigrants found in the population per generation. Numbers of migrants is then defined as M multiplies Θ.

4.4. Results

4.4.1 Genetic diversity

COI

In *Patella caerulea* 34 COI haplotypes were obtained from up to 29 variable nucleotide positions. Haplotype diversity (H_d) ranged between 0.423 ± 0.022 (standard error) and 0.841 ± 0.013. Nucleotide diversity (π_d) ranged between and 0.015 ± 0.003 and 0.052 ± 0.007 (Table 2). Mean π_d and H_d indices were not significantly different among locations (ANOVA: π_d $p = 0.671$ and H_d $p = 0.233$) and IN *vs.* OUT MPAs (ANOVA: π_d $p = 0.470$ and H_d $p = 0.698$).

In *P. rustica* mitochondrial COI showed 57 different haplotypes due to 48 polymorphic nucleotide positions. Haplotype diversity (H_d) ranged between 0.579 ± 0.028 and 0.846 ± 0.015, while nucleotide diversity (π_d) ranged between and 0.014 ± 0.003 and 0.033 ± 0.005 (Table 2). Mean π_d and H_d indices were significantly different among locations (ANOVA: π_d $p = 0.006$ and H_d $p = 0.011$) but not between IN and OUT MPAs (ANOVA: π_d $p = 0.776$ and H_d $p = 0.748$).

Microsatellites

No linkage disequilibrium was detected in any case (data not shown). Diversity indices in *P. caerulea* showed that allelic richness (*Ar*) ranged between 3.479 ± 0.202 in PORTOUT2 to 4.512 ± 0.069 in TAVIN2, while private allelic richness (*pAr*) ranged between 0.122 ± 0.051 in CABOUT2 to 0.374 ± 0.038 in PCROSOUT2. Observed heterozygosity (H_o) ranged between 0.367 ± 0.039 in PORTIN2 to 0.603 ± 0.055 in PCROSOUT1, while expected heterozygosity (H_e) ranged from 0.669 ± 0.214 in PORTOUT2 to 0.859 ± 0.028 in TAVIN2.

	Location (Lo)				Protection (Pr)				Lo × Pr				Res
Patella caerulea	MS	$F_{3,168}$	*p*		MS	$F_{1,3}$	*p*		MS	$F_{3,168}$	*p*		MS
H_d	0.5681	65.63	0	***	0.0746	0.23	0.6632	ns	0.322	37.2	0	***	0.0087
π_d	0.0021	2.47	0.064	ns	0.0019	0.44	0.5537	ns	0.0043	5.13	0.002	**	0.0008
Patella rustica	MS	$F_{3,168}$	*p*		MS	$F_{1,3}$	*p*		MS	$F_{3,168}$	*p*		MS
H_d	0.3729	42.86	0	***	0.0224	0.95	0.4022	ns	0.0237	2.72	0.0461	*	0.0087
π_d	0.0017	4.63	0.0039	**	0.0001	0.21	0.6758	ns	0.0004	1.22	0.3038	ns	0.0004

Significant levels were indicated by the following symbols: ns = not significant, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

Table 2. Mitocondrial DNA diversity indices for the species *P. caerulea* and *P. rustica* (n = number of individuals, H = number of haplotypes, H_d = mean haplotype diversity, π_d = mean nucleotide diversity, SD = standard deviation). Negative values means heterozygote excess.

Highly significant multilocus deviations from HW proportions were observed in all samples after FDR corrections (Table 4.3). Multilocus estimates of F_{IS} ranged from 0.249 ± 0.062 (PORTIN2) to 0.557 ± 0.067 (CABIN1), showing in all cases heterozygote deficiencies. Heterozygote deficit could be related to the presence of null alleles. Estimated null allele frequencies (r) across samples ranged between 0.012 ± 0.019 (Pru15) to 0.323 ± 0.066 (Pc36).

In the case of *P. rustica*, allelic richness ranged between 2.823 ± 0.173 in PORTOUT2 to 3.487 ± 0.114 in PCROSIN2, while private allelic richness ranged between 0.150 ± 0.008 in PORTOUT1 to 0.618 ± 0.042 in TAVOUT2. Observed heterozygosity ranged between 0.348 ± 0.038 in TAVIN2 to 0.690 ± 0.067 in TAVIN1, while expected heterozygosity ranged from 0.634 ± 0.230 in PORTOUT2 to 0.751 ± 0.016 in CABIN1. Significant deviations from HW equilibrium were observed in all samples and at all microsatellites loci expressed as heterozygosity deficiency. In fact, all the F_{IS} values are high and positive and ranged from 0.404 ± 0.046 (TAVOUT1) to 0.501 ± 0.084 in CABIN2 and PORTOUT2 (Table 4.4). Estimated null allele frequencies (r) across samples ranged between 0.040 ± 0.061 (Pc11) to 0.257 ± 0.060 (Pru5).

For both species, ANOVA tests on microsatellite diversity indices did not reveal significant differences among locations and IN *vs.* OUT MPAs (Table S.4.1, Supplementary content).

4.4.2 Genetic structure and connectivity patterns

The haplotype network of *Patella caerulea* presented a star-like shape, with three main haplotypes (H34, H18, H10) present in all sites (except for H10 in CABOUT1), and several private and low frequency haplotypes (Figure 4.2a). Pairwise F_{ST} showed significant genetic differentiation between CABOUT1 and PCROSIN1 and between CABOUT1 and PORTOUT1 after FDR correction (F_{ST} = 0.057, p = 0.0098 and F_{ST} = 0.1121 p = 0.0013, respectively; Table 4.5).

Species	Location	Protection	Site code	N	Ar ± se	pAr ± se	H_o ± se	H_e ± se	F_{IS} ± se
P. caerulea	Cabo de Palos	Protected	CABIN1	25	3.752 ± 0.191	0.166 ± 0.032	0.407 ± 0.048	0.749 ± 0.031	0.557 ± 0.067
			CABIN2	24	4.310 ± 0.119	0.183 ± 0.044	0.464 ± 0.040	0.740 ± 0.044	0.482 ± 0.083
		Unprotected	CABOUT1	24	4.310 ± 0.173	0.144 ± 0.047	0.472 ± 0.050	0.754 ± 0.111	0.469 ± 0.065
			CABOUT2	24	4.191 ± 0.174	0.122 ± 0.051	0.401 ± 0.047	0.742 ± 0.136	0.488 ± 0.079
	Port Cros	Protected	PCROSIN1	22	4.269 ± 0.160	0.214 ± 0.015	0.468 ± 0.039	0.767 ± 0.108	0.510 ± 0.058
			PCROSIN2	24	4.449 ± 0.092	0.270 ± 0.020	0.391 ± 0.049	0.838 ± 0.051	0.376 ± 0.065
		Unprotected	PCROSOUT1	24	3.757 ± 0.184	0.254 ± 0.111	0.603 ± 0.055	0.819 ± 0.185	0.492 ± 0.060
			PCROSOUT2	22	4.394 ± 0.103	0.374 ± 0.038	0.430 ± 0.050	0.835 ± 0.073	0.452 ± 0.042
	Portofino	Protected	PORTIN1	24	4.235 ± 0.218	0.325 ± 0.040	0.514 ± 0.045	0.814 ± 0.154	0.328 ± 0.080
			PORTIN2	24	4.191 ± 0.145	0.252 ± 0.651	0.367 ± 0.039	0.812 ± 0.099	0.249 ± 0.062
		Unprotected	PORTOUT1	23	4.028 ± 0.148	0.257 ± 0.028	0.544 ± 0.024	0.778 ± 0.164	0.357 ± 0.063
			PORTOUT2	24	3.479 ± 0.202	0.369 ± 0.103	0.384 ± 0.025	0.669 ± 0.214	0.365 ± 0.069
	Tavolara	Protected	TAVIN1	24	4.033 ± 0.138	0.260 ± 0.035	0.417 ± 0.045	0.798 ± 0.093	0.507 ± 0.033
			TAVIN2	16	4.512 ± 0.069	0.297 ± 0.046	0.405 ± 0.053	0.859 ± 0.028	0.447 ± 0.082
		Unprotected	TAVOUT1	24	4.136 ± 0.152	0.253 ± 0.008	0.465 ± 0.031	0.801 ± 0.133	0.552 ± 0.087
			TAVOUT2	24	3.961 ± 0.138	0.276 ± 0.047	0.470 ± 0.055	0.778 ± 0.125	0.551 ± 0.074

Table 4.3. Microsatellite diversity indices for the species *P. caerulea* (Ar =allelic richness, pAr = private allele richness, H_e = expected heterozygosity, F_{IS} = inbreeding coefficient, SD = standard deviation)

Species	Location	Protection	Site code	N	$Ar \pm$ se	$pAr \pm$ se	$H_o \pm$ se	$H_e \pm$ se	$F_{IS} \pm$ se
P. rustica	Cabo de Palos	Protected	CABIN1	25	3.318 ± 0.082	0.525 ± 0.073	0.420 ± 0.054	0.751 ± 0.016	0.466 ± 0.065
			CABIN2	24	2.911 ± 0.118	0.477 ± 0.079	0.388 ± 0.055	0.686 ± 0.162	0.501 ± 0.084
		Unprotected	CABOUT1	24	3.048 ± 0.108	0.588 ± 0.021	0.399 ± 0.042	0.704 ± 0.143	0.488 ± 0.053
			CABOUT2	24	2.968 ± 0.129	0.374 ± 0.191	0.399 ± 0.058	0.672 ± 0.188	0.442 ± 0.093
	Port Cros	Protected	PCROSIN1	22	3.037 ± 0.065	0.332 ± 0.028	0.509 ± 0.058	0.643 ± 0.090	0.457 ± 0.080
			PCROSIN2	24	3.487 ± 0.114	0.386 ± 0.072	0.367 ± 0.024	0.649 ± 0.113	0.436 ± 0.025
		Unprotected	PCROSOUT1	24	2.876 ± 0.065	0.355 ± 0.016	0.558 ± 0.054	0.673 ± 0.090	0.442 ± 0.075
			PCROSOUT2	22	3.056 ± 0.077	0.252 ± 0.145	0.465 ± 0.053	0.678 ± 0.087	0.438 ± 0.080
	Portofino	Protected	PORTIN1	24	3.077 ± 0.016	0.268 ± 0.038	0.565 ± 0.031	0.700 ± 0.127	0.499 ± 0.059
			PORTIN2	24	3.077 ± 0.085	0.265 ± 0.045	0.448 ± 0.057	0.706 ± 0.092	0.486 ± 0.101
		Unprotected	PORTOUT1	23	3.231 ± 0.086	0.150 ± 0.008	0.447 ± 0.020	0.744 ± 0.045	0.454 ± 0.103
			PORTOUT2	24	2.823 ± 0.173	0.186 ± 0.117	0.559 ± 0.045	0.634 ± 0.230	0.501 ± 0.089
	Tavolara	Protected	TAVIN1	24	3.010 ± 0.129	0.597 ± 0.043	0.690 ± 0.067	0.686 ± 0.187	0.486 ± 0.097
			TAVIN2	16	3.182 ± 0.147	0.593 ± 0.054	0.348 ± 0.038	0.707 ± 0.134	0.409 ± 0.053
		Unprotected	TAVOUT1	24	3.181 ± 0.057	0.590 ± 0.045	0.597 ± 0.036	0.731 ± 0.077	0.404 ± 0.046
			TAVOUT2	24	3.123 ± 0.119	0.618 ± 0.042	0.545 ± 0.053	0.716 ± 0.135	0.436 ± 0.082

Table 4.4. Microsatellite diversity indices for the species *P. rustica* (*Ar* =allelic richness, *pAr* = private allele richness, H_e = expected heterozygosity, F_{IS} = inbreeding coefficient, SD = standard deviation)

	CABIN1	CABOUT1	PCROSIN1	PCROSOUT1	TAVIN1	TAVOUT1	PORTIN1	PORTOUT1
CABIN1		0.0137	-0.015	0.0248	0.0415*	0.0878*	0.0453*	-0.0038
CABOUT1	0.0068		-0.015	-0.0106	-0.0121	0.002	-0.0104	-0.0126
PCROSIN1	0.0137	0.0573*		-0.018	-0.0257	-0.0215	-0.0059	0.0011
PCROSOUT1	0.0212	0.0029	0.0016		-0.013	-0.0037	-0.0094	-0.0094
TAVIN1	-0.0107	-0.0178	0.0209	0.0049		-0.0171	-0.0106	0.0009
TAVOUT1	0.0031	0.0096	-0.0085	-0.0164	-0.0022		0.0018	0.0296
PORTIN1	-0.0056	0.0424	-0.0175	0.0071	0.0204	-0.0067		0.0097
PORTOUT1	0.0048	0.1121*	-0.0193	0.0527	0.0453	0.0204	-0.0185	

Table 4.5. Mitochondrial genetic differentiation Kotsakiozi et al., among sites for each species. Upper right represents the F_{ST} values for *P. rustica* whereas down left represents the F_{ST} values for *P. caerulea*. Significant values after FDR correction (p =0.013) are represented with *. Negative values means heterozygote excess.

In the haplotype network of *P. rustica*, two central haplotypes (H35 and H51) separated by one single mutation step and surrounded by several low frequency haplotypes, were found in all sites (Figure 2b). F_{ST} values were low but significant between CABIN1-PORTIN1 (F_{ST} = 0.0453, p = 0.010), CABIN1-TAVIN1 and CABIN1-TAVOUT1 (F_{ST} = 0.0415; p = 0.011 and F_{ST} = 0.0888; p = 0.004, respectively; Table 4.4).

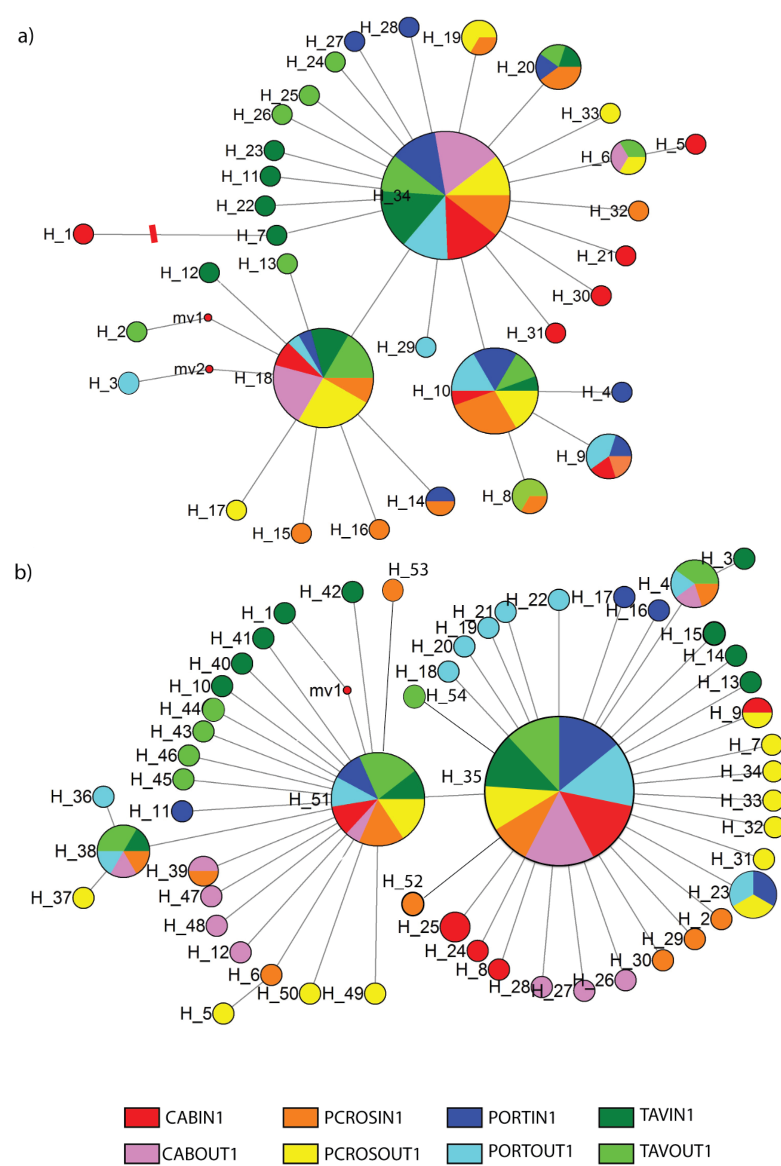


Figure 4.2. Unrooted mitochondrial haplotype network of the two species: *P. caerulea* (a) and *P. rustica* (b). Colours represent the sampling sites analysed. All haplotypes differ for 1 mutational step, except for the one of the red line with 2 mutational steps. Mv1 and mv2 represent median vectors.

In both species, patterns of mitochondrial differentiation were not explained by the isolation by distance model, and the analysis of molecular variance (AMOVA) did not reveal any significant pattern of differentiation among locations or inside and outside MPA.

Pairwise F_{ST} values according to microsatellite loci in *P. caerulea* ranged from 0.000 to 0.124. In spite of this, 97 out of 120 pairwise F_{ST} values were significant after FDR correction (Table S.2, Supplementary content). Pairwise F_{ST} in *P. rustica* showed comparable values ranging from 0.000 to 0.136 (Table S.3, Supplementary content) and 96 out of 120 pairwise values were significant after FDR.

F_{ST} estimates, as well as ENA F_{ST} estimates, of the microsatellite dataset gave similar results (*P. caerulea*: global F_{ST} = 0.039, global ENA F_{ST} = 0.034; *P. rustica:* global F_{ST} = 0.061, global ENA F_{ST} = 0.055). Overall, higher values of genetic differentiation were observed using D_{est}, rather than F_{ST} (Table S.2 and Table S.3, Supplementary content). Both estimators of population-pairwise differentiation were strongly correlated as shown by the Mantel test (r = 0.848, P < 0.001 for *P. caerulea* and r = 0.942, $p < 0.001$ for *P. rustica*).

According to AMOVA, genetic variance did not partition significantly among locations ($p = 0.478$ for *P. caerulea* and $p = 0.097$ for *P. rustica*) and more than 90% of the total variance was observed within sites.

Patterns of nuclear genetic differentiation were not explained by the isolation by distance model in *P. caerulea* ($p = 0.654$), while a slight significant correlation was observed in *P. rustica* ($p = 0.022$).

For *P. caerulea* the Bayesian clustering analysis including all the sites detected as optimal K, K = 2 nonetheless with certain levels of admixture between the two genetic clusters due to similar estimated proportion of membership of all the site to each of the 2 clusters (data not shown).

For *P. rustica* the Bayesian clustering analysis including all the sites detected as optimal K, K=4. Despite K=4, Structure plot seem differentiate CAB and PCROS locations from PORT and TAV (data not shown).

Within each location for both species, no significant effect IN and OUT MPA on the patterns of variation were observed according to AMOVAs (all $p > 0.05$).

For *P. caerulea*, no significant correlation was observed between genetic structuring and geographical distance within each location (P > 0.05). In *P. rustica* a pattern of isolation by distance was observed only among the sites of Port Cros in *P. rustica* ($p = 0.03$).

Even if with some differences, clustering of individuals for both species evidenced the presence of two genetic pools: one including individuals from IN1 and OUT1, and another including individual from IN2 and OUT2 in PCROS, CAB and TAV (Figure 4.3; Table S.4.4, Supplementary content). Nevertheless in *P. caerulea* the PCROSIN2 and TAVIN2 (located on the island) present individuals belonging to one unique cluster. Moreover, also PORTIN2 present an exclusive gene pool that differentiates this site from the others (Figure 4.3; Table S.4.4, Supplementary content).

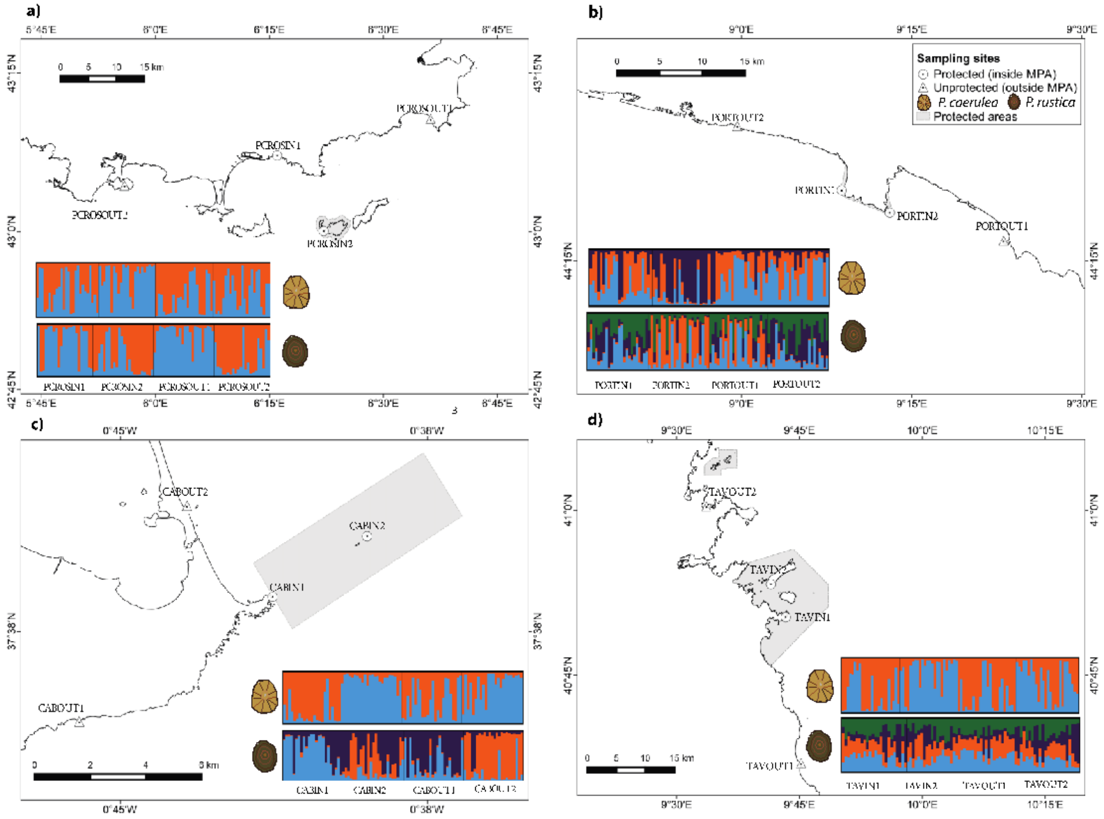


Figure 4.3. Results of the Structure analysis for *P. caerulea* in Port-Cros (a), Portofino (b), Cabo de Palos (c), and Tavolara (d) locations. Number represents the migration in round percentage. MPAs boundaries obtained from the World Database on Protected Areas; Mercator projection, Datum WGS 84.

Bayesian-based analysis using Migrate-n revealed that values of M were > 1 in all populations of both species, meaning that the effect of migration (*m*) is larger than the effect of mutation (μ). Moreover, migration was found to be almost bi-directional among sites within location. Nevertheless, *P. caerulea* showed a higher numbers of migrants compared to *P. rustica* in every location (Table S.4.5, Supplementary content).

4.5. Discussion

This study has analysed patterns of genetic variability and structuring in two *Patella* species across the north-western Mediterranean Sea, and inside and outside Marine Protected Areas. The three major results were: i) no significant differences on genetic diversity IN and OUT MPAs; ii) low genetic structure along the north-western Mediterranean coast in both species based on mitochondrial marker; iii) differences in patterns of genetic structure and connectivity within locations depending on species and local hydrodynamic features.

4.5.1. Patterns of genetic diversity within locations

Up to date only few studies have specifically studied patterns of genetic variability in populations inside and outside Marine Protected Areas in temperate benthic species (Bell, 2008a; McInerney et al., 2009b; Marti-Puig et al., 2013 for a review). For example in one of these studies made on the overfished and threatened pink abalone, an effect of protection on allelic diversity were observed (Munguía-Vega et al., 2015). In the two investigated *Patella* species no significant differences on genetic variability were observed for mitochondrial markers IN and OUT MPAs. No differences in genetic variability were detected neither according to the hyper-variable microsatellite markers, even if they have been effective in detecting variation in genetic diversity between populations of *P. caerulea* on natural and nearby artificial habitats (Fauvelot et al., 2009). In Tavolara–Punta Coda Cavallo, no significant variability within and outside MPA were observed not only in terms of genetic diversity but also in terms of abundance of individuals

(Ceccherelli et al., 2011). In fact, Ceccherelli et al. (2011) found that the rocky-shore gastropod assemblages were significantly influenced by the geographical siting of the locations (see also 4.5.3 paragraph).

These results could be related to the widespread distribution of *Patella* species and their high population density. Nevertheless, comparing the population density of *Patella* species inside and outside the other MPAs could provide data to support this hypothesis.

High heterozygosity deficiencies related to the presence of null alleles is a widespread phenomenon in Mollusca microsatellites (e.g. Pérez et al., 2007; Fauvelot et al., 2009). In the species and locations studied here, null allele frequencies observed were comparable to those obtained in previous studies (0.06-0.50 in *P. caerulea* (Fauvelot et al., 2009), 0.13-0.22 in *P. rustica* (Ribeiro, 2008). The high correlation between the two different estimators of genetic differentiation, the similar values of global estimates, and pairwise estimates assuming the presence of null alleles, suggest that the large heterozygote deficiencies may partially be due to a Wahlund effect, a common feature in limpets (Fauvelot et al., 2009). This seems also supported by the chaotic genetic structure observed at large scales (see paragraph 4.5.2).

4.5.2. Patterns of genetic structuring among locations

In both *Patella* species significant mitochondrial genetic differentiation was found between samples from Cabo de Palos and the other locations. F_{ST} estimates were low compared to those observed across known barriers to gene flow, as for *P. caerulea* across the Tyrrhenian and Ionian boundary (Villamor A, 2014) and for *P. rustica* across the Atlantic-Mediterranean transition (Sá- Pinto et al., 2010).

A study on the congeneric *P. ulyssiponensis* Gmelin, 1791 showed significant COI genetic differentiation between the north-western Mediterranean and the Alboran Sea (Cossu et al., 2015). In our case, genetic differentiation was observed nearer to the North Balearic front (Galarza et al., 2009; Schunter et al., 2011; Rossi et al., 2014). The Balearic basin is a transition zone between the Liguro-Provençal and the Algerian basins, which are characterised by contrasting

dynamic regimes and highly variable hydrological conditions (López García et al., 1994). The North Balearic front is known to act as a semi-permanent oceanographic barrier (Bouffard et al., 2010; Rossi et al., 2014) affecting genetic diversity and structuring of marine invertebrates (Costantini et al., 2007a; Mokhtar-Jamai et al., 2011). Observed genetic structuring could be related to long-term historical and oceanic processes, as suggested by the absence of a relationship between genetic diversity and geographical distance between locations. Extending the investigation in the Balearic transition region (e.g. Cabo de Palos, Medes Islands, Mallorca Island) to other invertebrates would allow testing this hypothesis.

The importance of oceanic processes in determining the genetic makeup of the two species of *Patella* in the north-western Mediterranean was also confirmed by the structure shown by the hypervariable markers. Overall, using microsatellite data, the pattern of distribution of genetic variability (as shown in F_{ST}, D_{est}, AMOVA and IBD analyses) showed a pattern of chaotic genetic patchiness in *P. caerulea* and a slight isolation by distance pattern in *P. rustica*. These differences could be explained by differences in the life history of the two species (see paragraph 4.3). Both genetic differentiation estimators gave consistent results albeit with low values of F_{ST} compared to those obtained using the actual measure of differentiation (D_{est}). Global F_{ST} estimates were higher than those obtained for *P. caerulea* and *P. rustica* sampled in the Adriatic Sea and the Eastern Atlantic Ocean, respectively (Fauvelot et al., 2009; Ribeiro et al., 2010). These discrepancies could be related to the high number of hydrodynamic provinces in which is divided the north-western Mediterranean, compared to the main and unidirectional currents flowing along the Atlantic coast of Iberia (Rossi et al., 2014). These hydrodynamic provinces are delimited by intense oceanic mesoscale structures such as jets, meanders, fronts and eddies that could affect the genetic structure of the populations. The geological history of the Adriatic Sea and its recent colonization after the Würm glaciation (~ 10,000 years ago) could also explain the differences observed in the genetic pattern of north-western Mediterranean *P. caerulea* populations compared to the Adriatic ones.

4.5.3. Genetic connectivity within locations

Microsatellites revealed low and complex patterns of genetic structuring within locations at spatial scales of thousands of meters. Nevertheless, an effect of migration among sites was also observed. Local currents are most likely to influence small-scale genetic connectivity. Indeed, in most of the locations migration patterns seem to follow the local surface currents. In Portofino the westward migration is in accordance with the direction of the main current, but also the high genetic differences in PORTIN2 observed in *P. caerulea* and the separation between the eastern and western part of Portofino Cape observed in *P. rustica* are supported by models of local circulation (Doglioli et al., 2004).

Dissimilarities in the connectivity patterns between *P. caerulea* and *P. rustica* at local scales may be driven by their population dynamics (e.g. density, age structure, sex ratio), life history traits (e.g. growth rate, fecundity, feeding), and competitive interactions with other species. On western Mediterranean rocky shores, limpets' distribution and abundance may change following seasonality and micro-scale topographic features (e.g. presence of crevices, slope of the substrate, wave exposure; Benedetti-Cecchi, 2001; Vaselli et al., 2008). As a consequence, limpets may alternate sharp reduction of population size with rapid increase in the number of individuals.

Closely related marine invertebrate species often display differences in patterns of genetic structuring (Kyle and Boulding, 2000; Becker et al., 2007). This variability has been related to the estimated levels of gene flow, and attributed to differences pelagic larval duration. Little is known about the reproductive biology of limpets however, along the Atlantic coast of Portugal, *P. rustica*, spawns once from September-October to December-January (Ribeiro, 2008), whereas *P. caerulea* has a longer reproductive period, with several spawning events occurring from September to April (Dodd, 1957; Bacci and Sella, 1970). According to this, limited larval output can be attributed to *P. rustica* (Ribeiro, 2008) compared to *P. caerulea* (Dodd, 1957; Côrte-Real et al., 1996; Sá- Pinto et al., 2010; Fauvelot et al., 2009), that could partially explain the differences observed in terms of diversity and differentiation.

Three of the investigated locations (with the exception of Portofino) included a sampling site on a protected island (i.e. CABIN2, PCROSIN2 and

TAVIN2; Figure 3 and Figure 4). For *P. caerulea*, patterns of genetic structuring within locations showed that populations sampled on islands were more isolated from the nearby mainland populations. This genetic differentiation between mainland and island populations were also observed in terms of large dissimilarities of abundance in other north-western Mediterranean locations (Benedetti-Cecchi et al., 2003) and within Tavolara– Punta Coda Cavallo MPA (Ceccherelli et al., 2011). These differences could be related to the intrinsic differences in the relevant processes operating in the two environments and to stochastic recruitment processes has already been described (Benedetti-Cecchi et al., 2003; Ceccherelli et al., 2011).

Genetic differentiation between the islands and the mainland has been described in two sessile and sedentary intertidal invertebrates: *Semibalanus balanoides* and *Nucella lapillus* (Bell, 2008b) observed that populations inhabiting islands are genetically differentiated from those on the nearby mainland, suggesting that larval flow from mainland to island sites may not be effective, compared to the larval flow among mainland populations.

Only few studies have been able to provide empirical evidence of effectiveness of MPAs in preserving genetic diversity and connectivity. Indeed a major goal for marine conservation policies is to establish networks of MPA, capable of maintaining and enhancing species resilience and ecosystem processes. High regional scale connectivity found in *Patella* species along the coasts of the western Mediterranean Sea, suggests that the established MPAs may potentially act as an effective network supporting long-term conservation at least for these species. Genetic monitoring through time (Schwartz et al., 2007) combined with demographic modelling would provide a powerful tool for monitoring and managing MPAs, based on empirical evidences of their effectiveness and limitations. This approach has been successfully implemented in assessment of management and conservation in the Indo-Pacific (Christianen et al., 2014). The present study could provide a starting point to include the genetic approach into the management of Mediterranean Marine Protected Areas.

4.6. Acknowledgments

Sampling was done in accordance with national laws, and authorizations were granted by local MPAs authorities. This study did not involve endangered or protected species. This research was funded by the European project 'Training Network for Monitoring Mediterranean Marine Protected Areas' (MMMPA: FP7-PEOPLE-2011-ITN; grant number 290056). Involved MPAs were associated partner of the MMMPA projects. PMP was supported by the MMPA project, as early stage researcher, and this study is part of her PhD. AV was supported by a research fellowship (Assegno di Ricerca) of the University of Bologna and 2010-11 PRIN project prot. 2010Z8HJ5M -Coastal bioconstructions: structures, functions, and management. Thanks to Roberto Buonomo and PopGen CCMAR discussion group for the Geographical distance R script. Thanks to Augusto Navone, Jose Antonio García-Chartón, Ramón García, José Pereguiñez, Marco Palma and Ubaldo Pantaleo for their assistance during the sampling.

4.7. Supplementary materials

	Location (Lo)				Protection (Pr)				Lo × Pr				Site (Lo × Pr)				Res
Patella caerulea	MS	$F_{3,8 \text{ or } 88\ (*)}$	p		MS	$F_{1,3}$	p		MS	$F_{3,8 \text{ or } 88\ (*)}$	p		MS	$F_{8,80}$	p		MS
Ar	0.4096	0.7427	0.5295	ns	0.5654	2.5118	0.2112	ns	0.2251	0.4081	0.7475	ns	pooled			ns	0.5515
pAr	0.093	2.049	0.1128	ns	0.0001	0.0069	0.939	ns	0.0131	0.2886	0.8335	ns	pooled			ns	0.0454
H_e	0.0101	0.7788	0.5089	ns	0.0143	1.4612	0.3133	ns	0.0098	0.7593	0.5199	ns	pooled			ns	0.0129
F_{is}	0.0144	0.1375	0.9374	ns	0.0024	0.4141	0.5657	ns	0.0057	0.0543	0.9832	ns	pooled			ns	0.1045
Patella rustica	MS	$F_{3,8 \text{ or } 72\ (*)}$	p		MS	$F_{1,3}$	p		MS	$F_{3,8 \text{ or } 72\ (*)}$	p		MS	$F_{8,64}$	p		MS
Ar	0.0265	0.1033	0.9579	ns	0.1974	1.8084	0.2713	ns	0.1092	0.4255	0.7353	ns	pooled			ns	0.2566
pAr	0.0209	0.1522	0.9254	ns	0.0612	7.2464	0.0743	ns	0.0084	0.0616	0.9786	ns	0.137	1.6328	0.1331	ns	0.0839
H_e	0.0026	0.2239	0.8795	ns	0.0049	0.7984	0.4374	ns	0.0062	0.5226	0.6681	ns	pooled			ns	0.0118
F_{is}	0.1459	1.1546	0.3331	ns	0.0748	3.3975	0.1625	ns	0.022	0.1742	0.9136	ns	pooled			ns	0.1264

Significant levels were indicated by the following symbols: ns = not significant, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

(*) if nested factor is pooled, the denominator is the residual

Table S4.1. Summary of ANOVA tests on rarefied allele richness (Ar), private allelic richness (pAr), expected heterozygosity (He) and inbreeding coefficient Gleick et al., in Patella caerulea and Patella rustica according to location, protection and site factors. pAr values were transformed by square root and Cochran's C tests were not significant after transformations.

	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**	**S13**	**S14**	**S15**	**S16**
S1		**0.107**	**0.038**	**0.136**	**0.035**	0.026	**0.048**	0.002	**0.078**	**0.070**	0.028	**0.118**	**0.064**	**0.053**	**0.081**	**0.043**
S2	**0.124**		**0.044**	**0.047**	**0.077**	**0.096**	**0.094**	**0.074**	**0.051**	**0.073**	**0.058**	**0.056**	**0.105**	**0.094**	**0.109**	**0.094**
S3	0.010	**0.082**		**0.104**	**0.038**	**0.034**	0.012	**0.050**	**0.082**	0.006	0.018	**0.113**	**0.077**	**0.075**	**0.087**	**0.053**
S4	**0.098**	**0.037**	**0.058**		**0.103**	**0.114**	**0.131**	**0.086**	**0.058**	**0.126**	**0.088**	**0.062**	**0.088**	**0.120**	**0.099**	**0.119**
S5	**0.035**	**0.041**	0.016	**0.042**		**0.042**	**0.027**	-0.005	**0.032**	**0.061**	0.011	**0.059**	**0.048**	0.034	**0.057**	0.017
S6	**0.122**	**0.094**	**0.087**	**0.048**	**0.059**		**0.054**	0.017	**0.067**	**0.055**	0.022	**0.102**	**0.065**	**0.048**	**0.060**	**0.049**
S7	**0.044**	**0.092**	0.007	**0.084**	**0.028**	**0.122**		**0.054**	**0.083**	0.024	0.021	**0.118**	**0.086**	**0.082**	**0.098**	**0.034**
S8	**0.034**	**0.039**	0.018	**0.046**	-0.008	**0.075**	**0.031**		0.008	**0.092**	0.019	**0.038**	0.019	0.027	**0.028**	0.021
S9	**0.060**	**0.106**	**0.053**	**0.064**	**0.055**	**0.086**	**0.076**	**0.056**		**0.082**	**0.034**	0.004	**0.059**	**0.036**	**0.054**	**0.030**
S10	**0.082**	**0.103**	**0.052**	**0.074**	**0.049**	**0.083**	**0.068**	**0.057**	**0.037**		**0.034**	**0.112**	**0.109**	**0.097**	**0.107**	**0.054**
S11	**0.034**	**0.073**	**0.029**	**0.055**	0.013	**0.068**	**0.045**	0.009	0.019	**0.046**		**0.064**	**0.040**	0.023	**0.036**	0.024
S12	**0.034**	**0.074**	**0.027**	**0.050**	0.014	**0.045**	**0.057**	0.019	**0.041**	**0.045**	0.023		**0.060**	**0.051**	**0.063**	**0.060**
S13	**0.054**	**0.051**	**0.034**	**0.045**	0.013	**0.059**	**0.043**	0.019	**0.044**	**0.041**	**0.029**	0.011		0.021	**0.026**	**0.062**
S14	**0.097**	**0.035**	**0.077**	**0.040**	**0.046**	**0.077**	**0.108**	**0.030**	**0.103**	**0.105**	**0.059**	**0.060**	**0.057**		**0.031**	0.030
S15	**0.040**	**0.068**	0.016	**0.061**	0.007	**0.085**	0.013	0.011	**0.067**	**0.072**	**0.034**	**0.030**	0.014	**0.076**		**0.079**
S16	**0.052**	**0.098**	**0.047**	**0.076**	**0.044**	**0.071**	**0.064**	**0.042**	0.013	**0.027**	0.021	0.023	0.021	**0.086**	**0.058**	

Table S4.2. Pairwise FST values for microsatellites for Patella caerulea (down left) and for P. rustica (upper right). Significant values after FDR correction ($p = 0.0093$) are represented in bold. S1=CABIN1; S2=CABIN2; S3=CABOUT1; S4=CABOUT2; S5= PCROSIN1; S6=PCROSIN2; S7=PCROSOUT1; S8=PCROSOUT2; S9=PORTIN1; S10=PORTIN2; S11=PORTOUT1; S12=PORTOUT2; S13=TAVIN1; S14=TAVIN2; S15=TAVOUT1; S16=TAVOUT2.

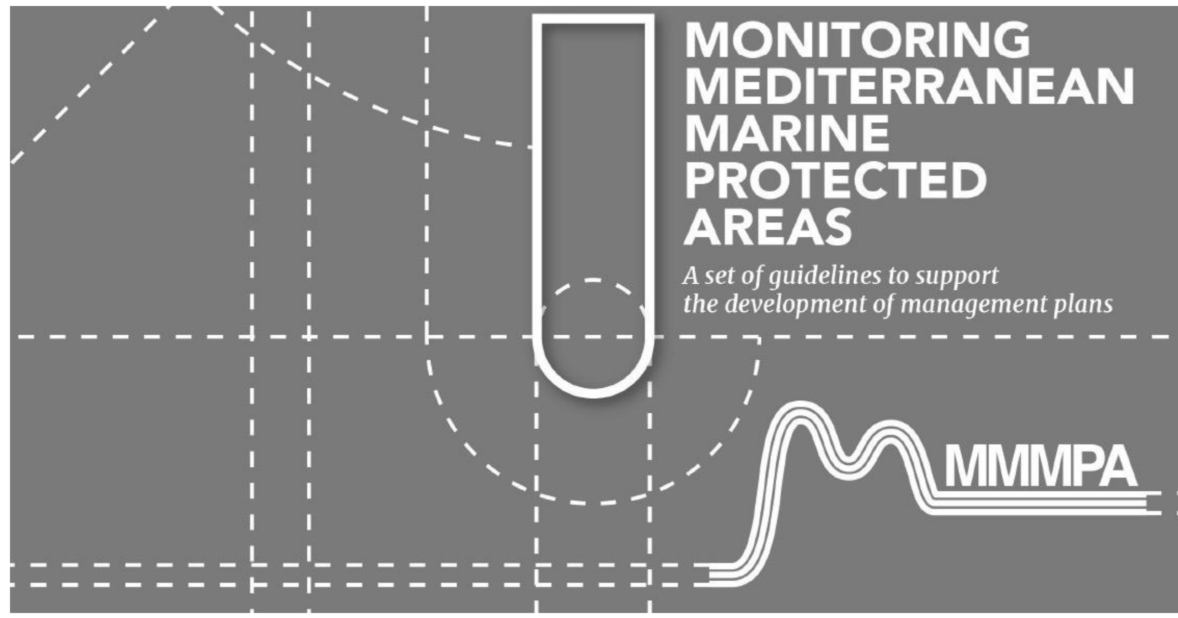
Species	Location	K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
P. caerulea	**CAB**	1	20	-2729.410	0.836	NA	NA	NA
		2	**20**	**-2642.300**	**5.598**	**87.110**	**44.960**	**8.031**
		3	20	-2600.150	25.442	42.150	9.965	0.392
		4	20	-2567.965	64.608	32.185	14.960	0.232
		5	20	-2550.740	56.021	17.225	40.820	0.729
		6	20	-2574.335	131.714	23.595	NA	NA
	PCROS	1	20	-2716.560	0.716	NA	NA	NA
		2	20	-2658.415	6.962	58.145	50.880	7.308
		3	20	-2651.150	98.012	7.265	23.890	0.244
		4	20	-2667.775	74.566	16.625	60.205	0.807
		5	20	-2624.195	47.440	43.580	52.105	1.098
		6	20	-2632.720	74.001	-8.525	NA	NA
	PORT	1	20	-2304.090	0.624	NA	NA	NA
		2	20	-2255.490	6.586	48.600	68.520	10.404
		3	20	-2275.410	26.006	19.920	17.595	0.677
		4	20	-2312.925	98.529	37.515	98.385	0.999
		5	20	-2252.055	73.254	60.870	51.270	0.700
		6	20	-2242.455	47.967	9.600	NA	NA
	TAV	1	20	-2698.580	0.824	NA	NA	NA
		2	20	-2686.015	44.935	12.565	81.465	1.813
		3	20	-2591.985	16.643	94.030	103.995	6.249
		4	20	-2601.950	82.683	-9.965	54.425	0.658
		5	20	-2666.340	76.158	64.390	96.455	1.267
		6	20	-2634.275	63.949	32.065	NA	NA

Table S4.3. Evanno table output by structure harvester per species per location for *P.caerulea*.

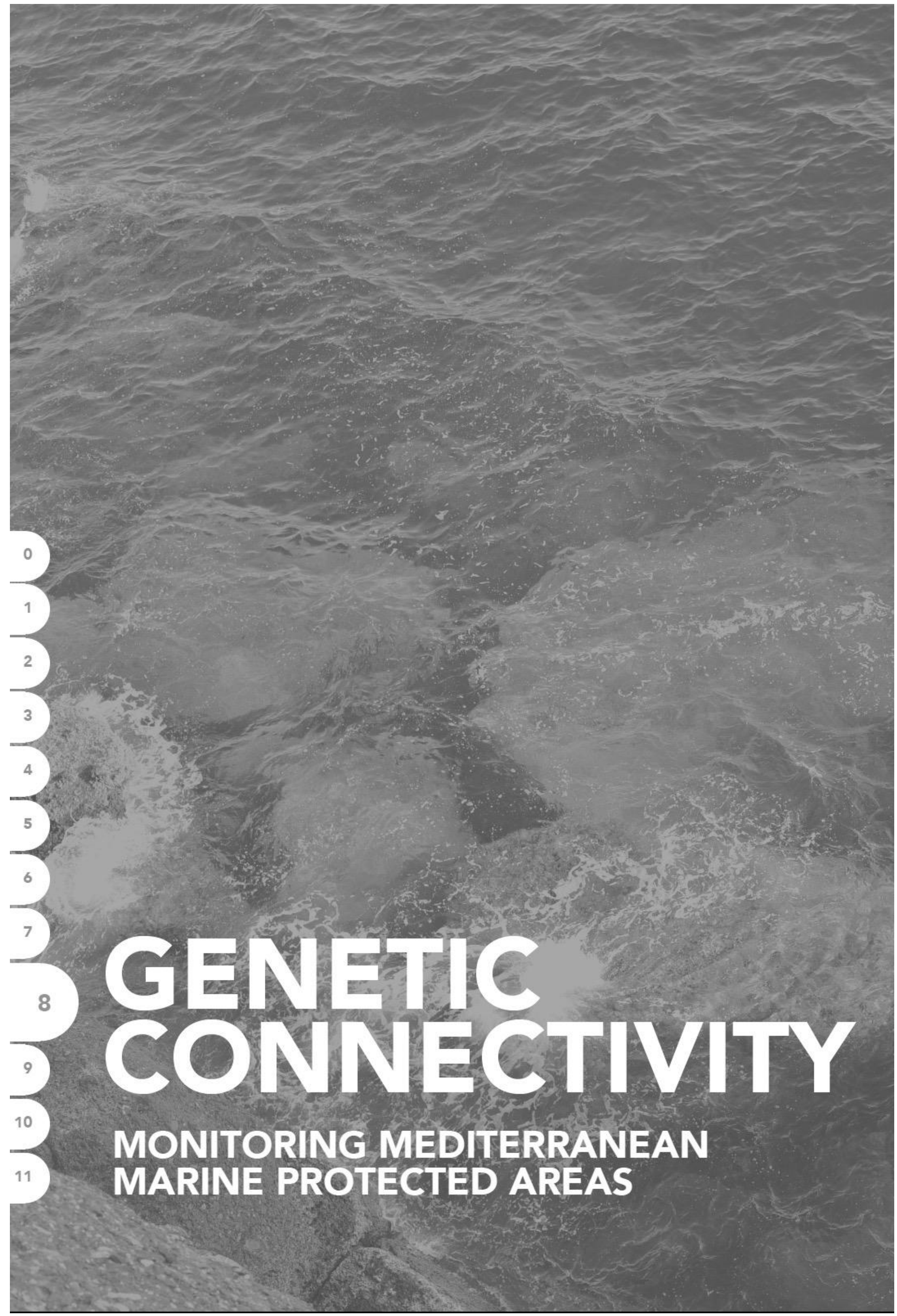
Species	Location	K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
P. rustica	**CAB**	1	20	-1778.105	0.733	NA	NA	NA
		2	20	-1664.600	4.515	113.505	62.555	13.854
		3	20	-1613.650	16.594	50.950	49.530	2.985
		4	20	-1612.230	29.669	1.420	36.290	1.223
		5	20	-1647.100	44.266	-34.870	48.185	1.089
		6	20	-1633.785	36.988	13.315	NA	NA
	PCROS	1	20	-1573.320	0.642	NA	NA	NA
		2	20	-1500.485	10.097	72.835	40.935	4.054
		3	20	-1468.585	17.010	31.900	18.490	1.087
		4	20	-1418.195	7.159	50.390	57.360	8.013
		5	20	-1425.165	19.421	-6.970	8.735	0.450
		6	20	-1440.870	28.779	-15.705	NA	NA
	PORT	1	20	-1672.670	0.590	NA	NA	NA
		2	20	-1600.655	4.142	72.015	70.250	16.962
		3	20	-1598.890	26.589	1.765	13.510	0.508
		4	20	-1583.615	34.806	15.275	6.990	0.201
		5	20	-1561.350	35.197	22.265	87.195	2.477
		6	20	-1626.280	97.331	-64.930	NA	NA
	TAV	1	20	-1571.080	0.484	NA	NA	NA
		2	20	-1530.995	5.330	40.085	21.280	3.993
		3	20	-1512.190	26.849	18.805	19.210	0.715
		4	20	-1512.595	16.619	-0.405	38.405	2.311
		5	20	-1551.405	58.443	-38.810	14.885	0.255
		6	20	-1605.100	72.474	-53.695	NA	NA

Table S4.4. Evanno table output by structure harvester per species per location for *P.rustica.*

CHAPTER V: GUIDELINES FOR THE DESIGN OF MARINE PROTECTED AREAS, USING GENETIC CONNECTIVITY AND DIVERSITY TOOLS



Publication note: The content of the following chapter has been published as: *Marti-Puig, P., Calò, A., Costantini, F., Villamor, A., Abbiati, M., Ponti, M., García-Charton, J.A., 2016. Genetic connectivity and diversity as a tool to assess the effectiveness of Marine Protected Areas, in: MMMPA Supervisory Board (Ed.), Monitoring Mediterranean Marine Protected Areas: A set of guidelines to support the development of management plans. Deliverable of the MMMPA European project (FP7-PEOPLE-2011-ITN g.a. no.: 290056), Ancona, pp. 81-87.*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GENETIC CONNECTIVITY
MONITORING MEDITERRANEAN MARINE PROTECTED AREAS

Why monitor genetic connectivity and diversity

The design and management of Marine Protected Areas (MPAs) and MPA networks should consider spatial patterns of species distribution and connectivity among populations (Green et al., 2014). Connectivity is the exchange of individuals among populations through the passive transport and/or active movement of individuals at whatever life stage (i.e. gametes, larvae, juveniles, sub-adults and adults) (Cowen and Sponaugle, 2009). Beside its importance in MPA design, connectivity is a fundamental aspect to consider when evaluating the status of existing MPAs and their ability to participate in an effective network, since well-connected and highly diverse populations are more resilient to environmental changes and less subjected to face local extinctions (Kaplan et al., 2009; Planes S, 2009) (Figure 5.1).

Figure 5.1: Effect of connectivity and genetic diversity on the resilience of local populations.

From this perspective, the investigation of connectivity patterns can be used as a management tool, providing information on: Villamor A, 2014 the portion of individuals coming from protected populations retained within MPA borders, allowing assessment of the level of self-sustainment of populations living inside the MPA; (Villamor A, 2014). the amount of individuals exported from protected populations toward unprotected areas, that gives an estimate of the ability of a MPA to supply outer unprotected locations (Gleick et al., 2010). The strength and direction of the connections between a MPA and the other MPAs that indicates if a MPA is acting as a 'source' and/or 'sink' of propagules (i.e. eggs and larvae). All

this information can help managers assess the status of their MPAs, and to address specific management issues in order to improve and/or maintain MPA health and effectiveness.

Monitoring genetic connectivity in MPAs is important because allows to:

- Assess the level of self-sustainability of populations living inside the MPA.
- Estimate the ability of an MPA to supply outer fished locations.
- Know if a MPA is acting as a 'source' and/or 'sink' of propagules.

How to monitor genetic connectivity and diversity

Different methods can be used to assess connectivity patterns between populations of marine organisms: e.g. biophysical larval dispersal models, genetic analyses, chemical analysis of carbonatic structures (such as fish otoliths). Each method has its advantages and disadvantages and none are flawless for assessing connectivity patterns (Calò et al., 2013, Jones et al., 2009). However, in the context of MPA monitoring, genetic tools could be preferable as they permit assessment of connectivity patterns at different temporal and spatial scales, and are possibly non-lethal, allowing their application on endangered species and focal species (Calò et al., 2013, Marti-Puig et al., 2013). Moreover, they can be used to investigate diversity and connectivity patterns in a huge variety of marine organisms with standard approaches equally valid for all animal or plant taxa.

A general approach for the monitoring of connectivity patterns should take into account the characteristics of the monitored MPA but also a series of aspects that would allow us to have a representative sampling design (Figure 2). From this perspective, the number of sampling sites should be defined depending on the geographic extension of the study area. The distance among sites would depend on the MPA size, the geomorphological and environmental characteristics, and the target species (Marti-Puig et al., 2013). A replicated design with selection of two or more site inside and outside the MPA in order to evaluate MPA effectiveness. Specifically, for genetic analysis, at each site, 20-30 individuals per species should be collected, for instance, within an area of approximately 100 m., separated from 1-10 m apart in the case of sessile individuals or sampling from different shoals in

the case of fishes, in order to avoid clones or collection of closely related specimens (Bell, 2008a, Costantini et al., 2007a). A small amount of tissue is enough for genetic analysis, which usually can be extracted without harming or killing the individual. Samples should be preserved in 90% ethanol and maintained at 4 ºC until processing. Cost per unit area would depend on the species selected for the monitoring, and the type of analysis needed. Samples could be extracted and sent to a sequence facility with a relatively low cost (DNA sequencing cost around 200€ for 96 samples). Moreover, nowadays genetics is evolving very fast, and cheaper and faster analysis such as next generation sequencing are available (Csencsics et al., 2010).

Since connectivity patterns differ among species (Coleman et al., 2011), several species should be selected to better address MPA management issues (Marti-Puig et al., 2013), as well as, additional information, such as oceanographic current data and demographic data, should be integrated in connectivity studies, in order to better interpret the results.

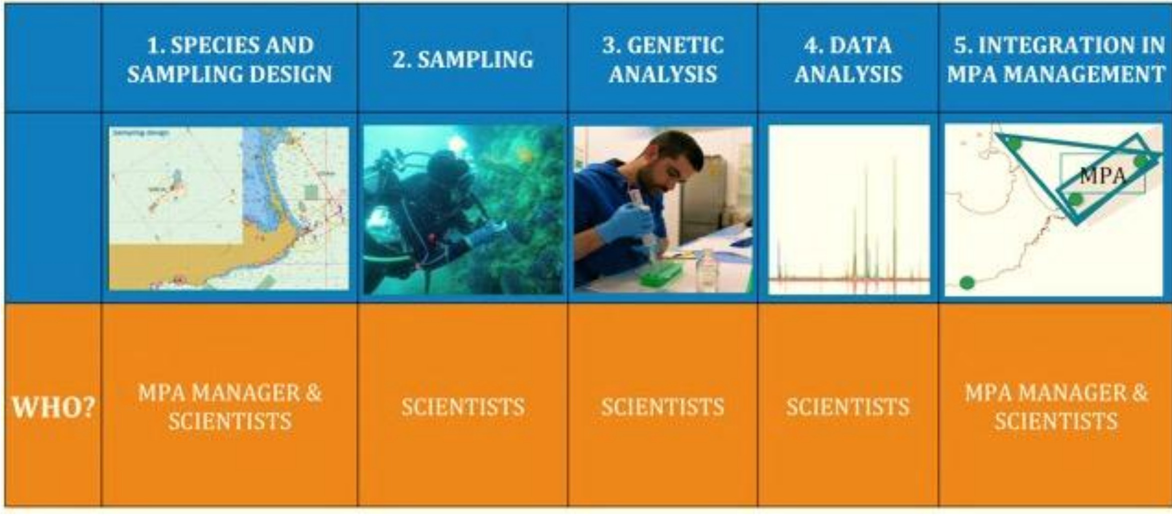
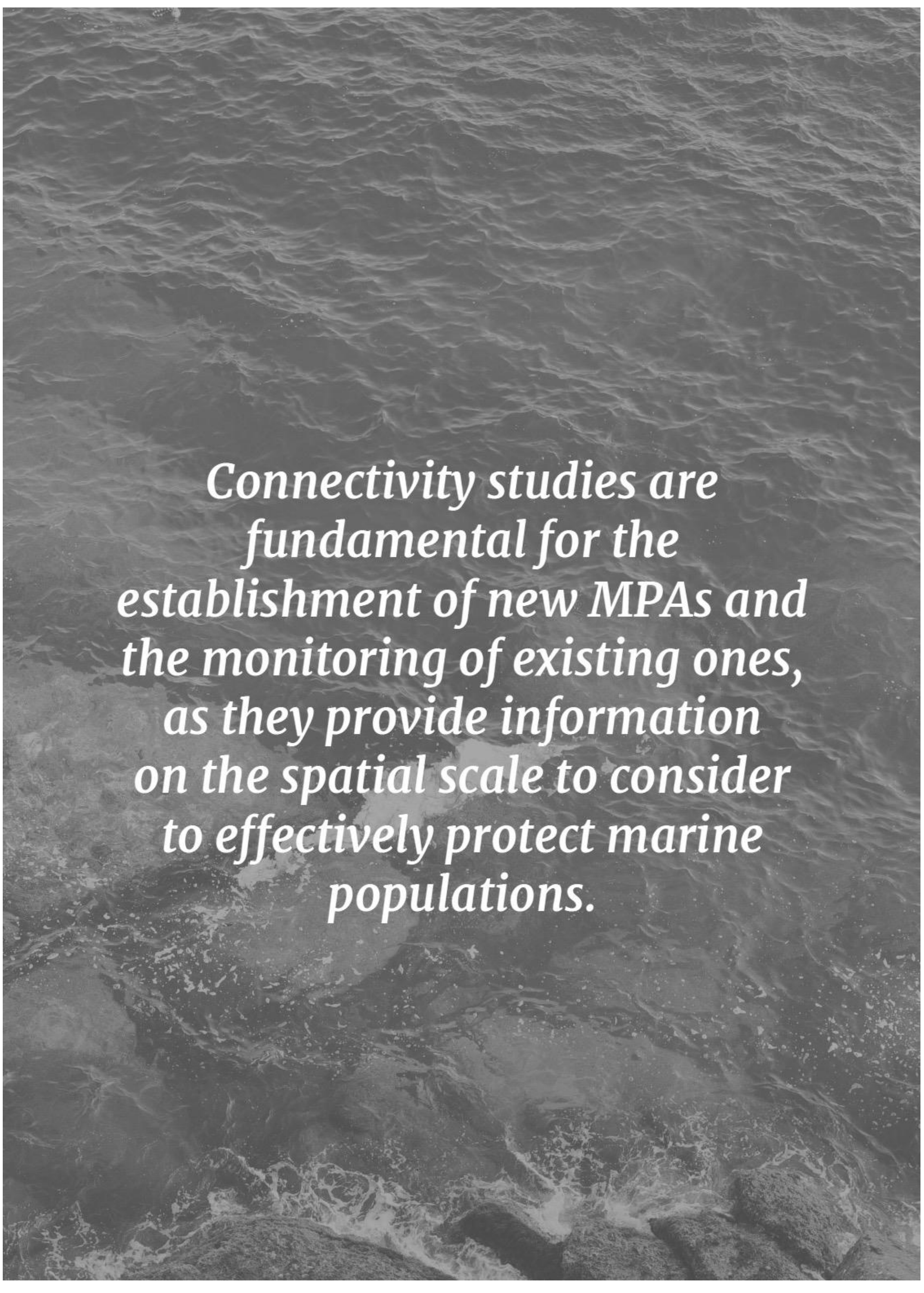


Figure 5.2: Schematic standard approach for gathering data on genetic connectivity and integrate them for the development of marine conservation strategies.


Connectivity studies are fundamental for the establishment of new MPAs and the monitoring of existing ones, as they provide information on the spatial scale to consider to effectively protect marine populations.

A case study on fishes: the saddled sea bream

The saddled sea bream (*Oblada melanura*) is an economically important species, widely distributed in Mediterranean coastal ecosystems. Although generally protected within Mediterranean MPAs, population genetic patterns of this species are currently unknown in the Western Mediterranean Sea. With this aim, the genetic structure of the saddled sea bream and the level of genetic connectivity between protected and unprotected populations was investigated, using a set of 11 microsatellite loci. Spatial patterns of population differentiation were assessed locally (50-100 km) and regionally (500- 1000 km), considering three MPAs of the Western Mediterranean Sea. All values of population differentiation (F_{ST} and Jost's D) were non-significant after Bonferroni correction, indicating that, at a relatively local spatial scale, protected populations were in general well connected with non-protected ones. On the other hand, at the regional scale, statistical analyses (i.e. discriminant analysis of principal components, AMOVA and STRUCTURE) revealed the presence of a subtle population structure that reflects the main oceanographic features (currents and barriers) of the study area (Figure 5.3).

This genetic pattern (population divergence in presence of high gene flow) could be a consequence of different processes acting at different spatial and temporal scales among which species dispersal capacity, the presence of admixed populations or large population size could play a major role. These results may have important implications for the conservation biology and fisheries management of saddled sea bream like other coastal fish, as spatial variability in connectivity patterns may promote long-term stability of fish populations. From this perspective, multi-scale patterns of genetic connectivity should be taken into account when future MPAs will be established in the western Mediterranean Sea, implementing the existing network.

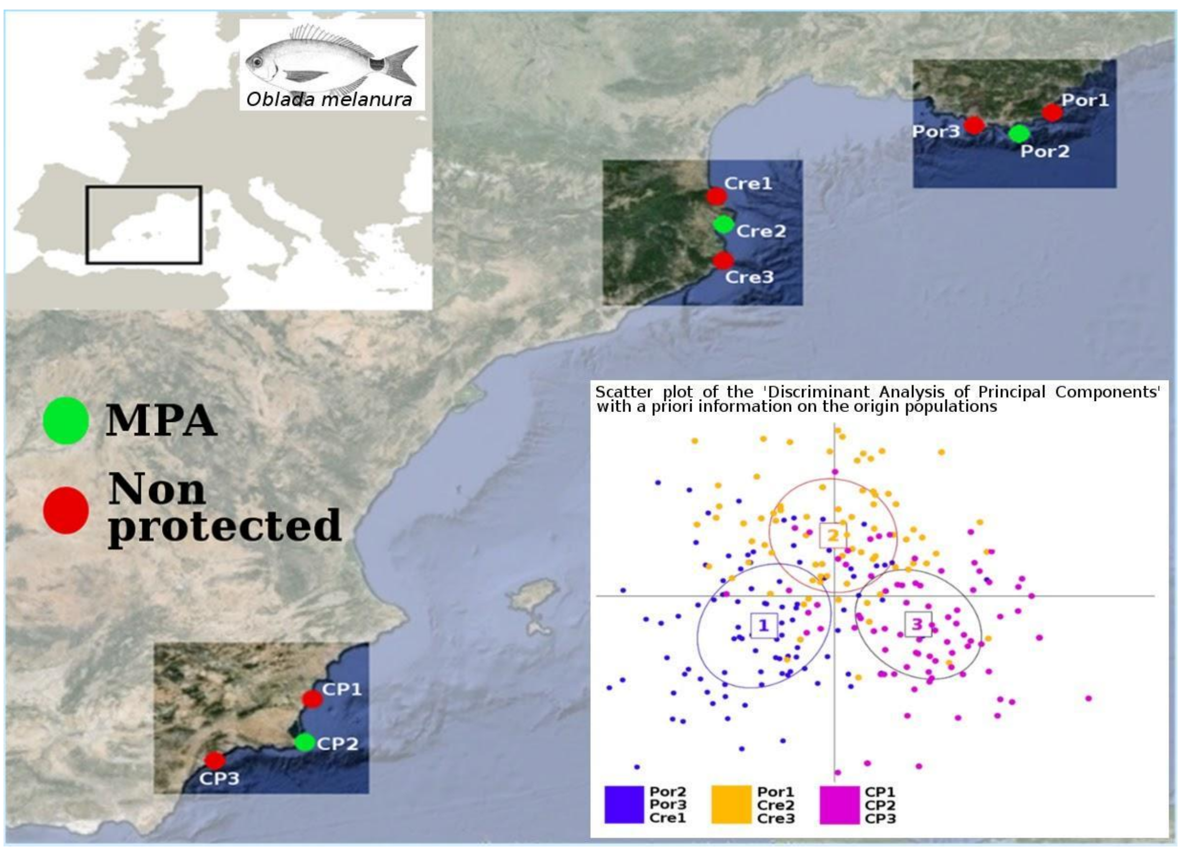


Figure 5.3: Study area and species. Down-right, scatterplot from DAPC analysis with information on original sampling locations.

A case study on intertidal invertebrates: the limpets

Limpets have a key ecological role in structuring rocky intertidal assemblages. Therefore their conservation is essential to protect these communities. Genetic variability and population connectivity of two widely distributed limpets (*Patella caerulea* and *P. rustica*) were analysed inside and outside four MPAs in the western Mediterranean Sea using mitochondrial and microsatellite markers. No effect of protection on genetic variability was observed in either species (Figure 5.4).

Mitochondrial marker reveals for both species limited genetic structure among MPAs in the north-western Mediterranean. Within each location, different patterns of genetic structure and connectivity were observed depending on the species and local hydrodynamic features (Figure 4). These and future genetic connectivity studies will help to MPA managers for the design of MPAs in order to enhance connectivity and genetic diversity that will increase the resilience of marine populations.

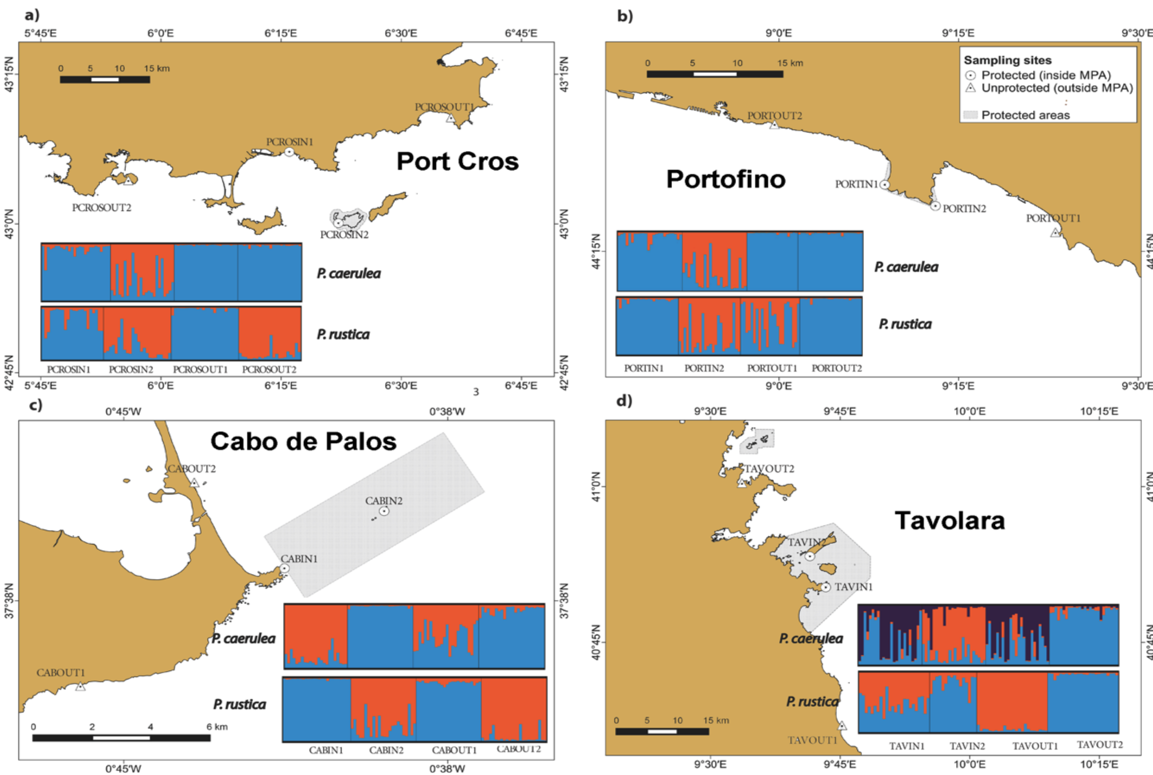


Figure 5.4: Possible results obtained by genetic analysis that will help to understand the populations structure and connectivity patterns.

Remarks

MPA design and monitoring based on connectivity assessment should take into account:

1) The knowledge of the biology and ecology of the model species, including:

- Life history traits, habitat preferences and behaviour

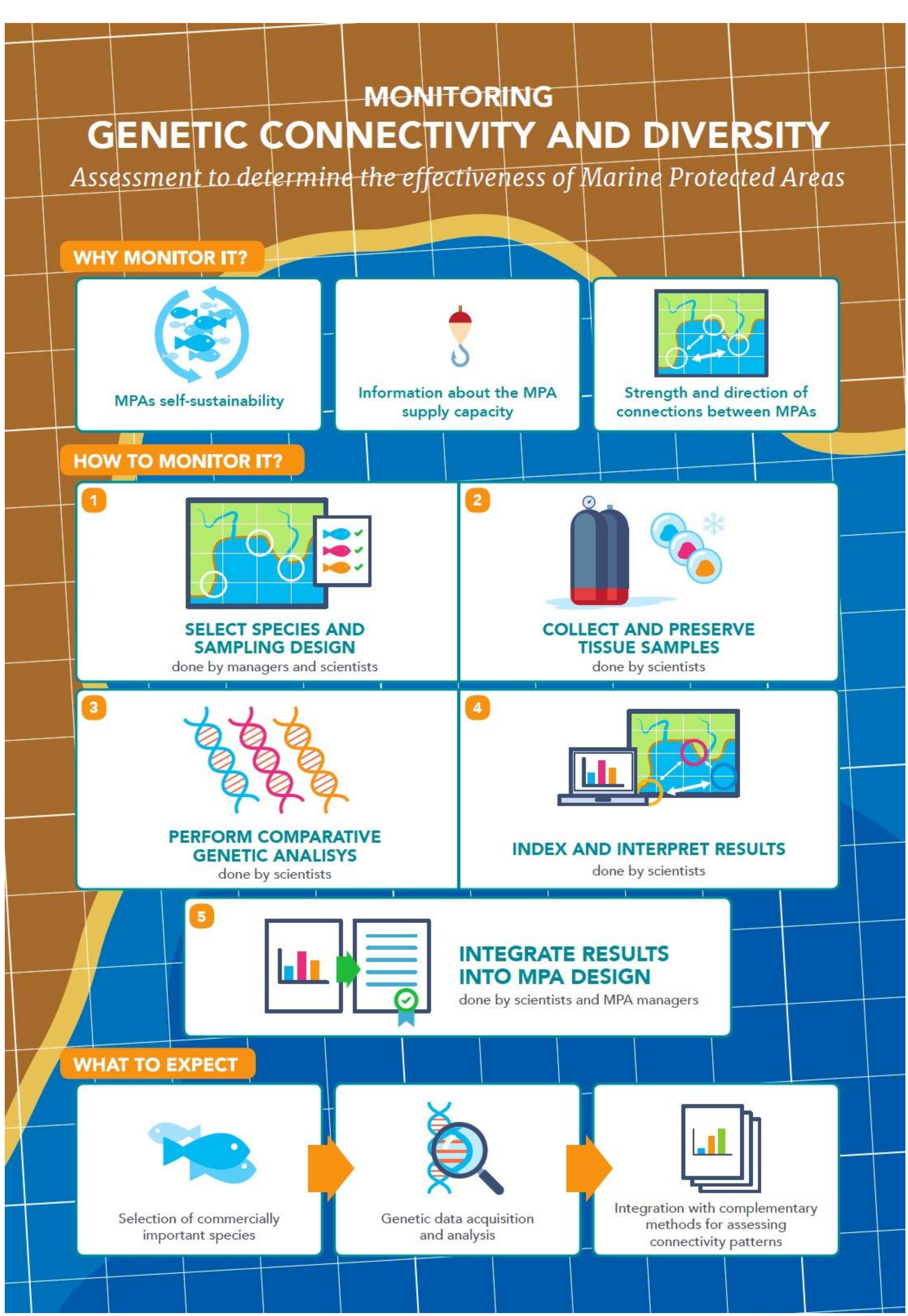
- Larval dispersal capability and movement characteristics

- Population genetic background

2) The environmental features in the area, including:

- Information on hydrodynamic patterns

- Information of the habitat characteristics



MONITORING
GENETIC CONNECTIVITY AND DIVERSITY
Assessment to determine the effectiveness of Marine Protected Areas
WHY MONITOR IT?
MPAs self-sustainability
Information about the MPA supply capacity
Strength and direction of connections between MPAs
HOW TO MONITOR IT?
1
SELECT SPECIES AND SAMPLING DESIGN
done by managers and scientists
2
COLLECT AND PRESERVE TISSUE SAMPLES
done by scientists
3
PERFORM COMPARATIVE GENETIC ANALISYS
done by scientists
4
INDEX AND INTERPRET RESULTS
done by scientists
5
INTEGRATE RESULTS INTO MPA DESIGN
done by scientists and MPA managers
WHAT TO EXPECT
Selection of commercially important species
Genetic data acquisition and analysis
Integration with complementary methods for assessing connectivity patterns

CHAPTER VI: GENERAL DISCUSSION

Port-Cros Marine Protected Area. Photo source: Patricia Marti-Puig

Over the past decades, due to the overexploitation of marine resources, there is an increasing need to regulate human activities and reach conservation objectives through the establishment of effective Marine Protected Areas (MPAs) (Agardy, 1994; Gaines et al., 2010). One of the main issues is that MPAs are not usually designated to take in account the biology or ecology of the marine species and their habitats. Deciding boundaries for management requires considerable information collected from a number of sources, including information about the diversity of the populations and how populations are connected within and beyond these boundaries. There is a need to develop and integrate adequate tools that can be applied and implemented to monitor MPA effectiveness. Within this context, the aim of this thesis was to develop a protocol for monitoring Marine Protected Areas by studying the morphology and genetics of two closely limpet species (*Patella rustica* and *Patella caerulea*) across MPAs in the Western Mediterranean sea.

In this thesis, I examined the steps needed to develop a sound monitoring: from the sampling design criteria, to morphological traits analysis, estimation of genetic diversity and connectivity, the potential integration of them into the MPA design. There are several considerations that MPA managers need to take into account when monitoring MPAs or MPA networks: 1) there is a need of planning effective studies for evaluating MPAs, requiring a sampling design that includes comparisons between protected and non-protected sites, comprising several species and different habitats; 2) genetic and morphometric tools need to be combined when species identification is challenging and to monitor morphological variability; 3) different genetic markers should be used (e.g. mitochondrial, microsatellites) to assess genetic structure and connectivity patterns at different temporal and spatial scales 4) it is necessary to integrate these tools into all the steps of MPA planning: from initial design to final monitoring of the effectiveness.

This thesis showed that morphometric and genetic tools are useful for identifying species, species variability and to evaluate structure and connectivity between and within MPAs. It also showed that biological patterns are different even between closely related species and also they depend strongly on the environment characteristics. Therefore, management needs should be studied on

a case by case basis, according to the conservation objectives of the MPA and the particular characteristics of the species of interest and habitat to be preserved. Nonetheless, this information has to be adequately integrated along with other data such as other social, economic and biological data (Marti-Puig,). Similar studies over long term have important implications, as climate change is expected to influence pelagic larval dispersal, and thus, connectivity patterns among marine populations (Andrello et al., 2015). Further challenges are to communicate effectively to Marine Protected Area managers the interpretation, integration and visualisation of these results.

Overall, this thesis provides a guideline for using morphometric and genetic tools to assess status of Marine Protected Area, and will help managers to address specific management issues in order to improve and/or maintain MPA health and effectiveness.

ANNEX: COMMUNICATION AND OUTREACH

ECSA54 conference. Foto source: http://www.mmmpa.eu/conferences.asp

In this Annex I included:

- Short animation movie

- Conference: ECSA54, Sesimbra, Portugal, 12-16 May 2014

- Conference: MMMPA, Ancona, Italy. 15 - 17 October, 2015

Short animation movie

Abstract: "Zoe and her adventures in the Mediterranean Sea" with the take home message "Protect and connect the oceans - Don't leave MPAs alone!", aims to inform young people about marine conservation and the importance of well-connected MPA network in the Mediterranean Sea. This short animation movie was created with the collaboration with my MMMPA colleagues in Italy. To promote the movie I have created a blog, film freeway page, facebook page, presented in conferences, social media and to several film competitions. Zoe and her adventures in the Mediterranean Sea took 1st place at the Emerging Filmmakers Competition at the 2016 Gray's Reef Ocean Film Festival.

Authors: Patrica Marti Puig, Vasiliki Markantonatou and Paula Andrea-Zapata. 3D animator: Kouvelis, Drawings of benthic organisms: Cristina Giogia di Camillo, Producer: ITN - Monitoring Mediterranean Marine Protected Areas (MMPA) (FP7/2007-2013) under Grant Agreement no.: 290056. Music director and composer: Pablo Villegas

Relevant links:

- Short-movie page: http://m.youtube.com/watch?v=bFhexhq6tGE

- Film-Freeway page: http://filmfreeway.com/projects/427314

- Zoe's blog: http://zoeadventures.tumblr.com/

- Facebook page: http://www.facebook.com/Zoe-adventures-in-the-Sea-797284323663020/?fref=ts

Screenings and prizes:

- Selected for screening in the Educational Program of SFIOFF. March 10th at 10am and 1pm and on Friday March 11th at 1pm 2016. http://oceanfilmfest.org/education/

Selected and presented for screening at the San Francisco International Ocean Film Festival 2016. http://oceanfilmfest.org/

1st place at the Emerging Filmmakers Competition at the 2016 Gray's Reef Ocean Film Festival.

Presented and selected for screening at the Gray's Reef Ocean Film Festival 2016.

Promoted in DAN Europe (Divers Alert Network Europe)

Promoted through the Spanish ministry website: http://www.reservasmarinas.net/rmarinasnews/not.ashx?n=6000000733

Presented at the Mediterranean Marine Protected Areas conference in Ancona from 15th to 17th October 2015

Conference: ECSA54, Sesimbra, Portugal, 12-16 May 2014

Population connectivity within and among Mediterranean MPAs: a case study using two closely related intertidal species (abstract).

P. Marti-Puig[1], M. Ponti[1], F. Costantini[1], A. Villamor[1], M. Abbiati[1,2]

[1] BiGeA & CIRSA, Università di Bologna, UO CoNISMa, Via S. Alberto 163, 48123 Ravenna, Italy.

[2] ISMAR, Consiglio Nazionale delle Ricerche - Istituto di Scienze Marine, Bologna, Italy.

Marine Protected Areas (MPAs) networks should be designed to protect species diversity and ensure long-term persistence of species. For achieving this purpose, MPAs should be efficient in terms of maintaining genetic diversity and connectivity at different spatio-temporal scales. Here, the efficiency of four western Mediterranean MPAs (Cabo de Palos, Port-Cros, Tavolara and Portofino) was assessed comparing the genetic variability of two widely distributed congeneric species of limpets (*Patella caerulea* and *Patella rustica*) in protected and nearby non-protected sites. Mitochondrial cytochrome oxidase c subunit 1 region was used for evaluating the connectivity among MPAs whereas the more variable microsatellite markers were used for evaluating connectivity patterns within and around each MPA. Genetic diversity differed among protected and non-protected sites for both species, with higher extent for *P. caerulea*. However, this "MPA effect" was not consistent in all locations. At geographic level, both species showed high genetic connectivity between Port-Cros, Tavolara and Portofino locations (including MPA and surrounding sites) with slight significant genetic differentiation with the most south-western location (Cabo de Palos). At local MPA level, no protection effect was observed in genetic diversity. Moreover within each MPA, different patterns of genetic structure and connectivity were observed depending on species and local environmental features. The results of this study suggest that multi-species and multi-scale management approaches are needed to evaluate the efficiency of MPAs.

Morphometric and genetic distinctness between two closely related species of limpets (*Patella rustica* and *Patella caerulea*) among Marine Protected Areas in the western Mediterranean sea (poster)

ECSA 54 - Coastal systems under change: tuning assessment and management tools
12-16 May 2014 Sesimbra, Portugal

MMMPA

Morphometric and genetic distinctness between two closely related species of limpets (*Patella rustica* and *Patella caerulea*) among Marine Protected Areas in the western Mediterranean Sea

Patricia Marti Puig[1], Massimo Ponti[1], Federica Costantini[1], Marco Abbiati[1,2]

1 - Dipartimento di Scienze Biologiche, Geologiche e Ambientali, University of Bologna, UO CoNISMa,Via S. Alberto 163, 48123 Ravenna, Italy
2 - ISMAR, Consiglio Nazionale delle Ricerche - Istituto di Scienze Marine, Bologna, Italy

Introduction

Limpets of the genus *Patella* show a high phenotypic plasticity often related to environmental conditions (Hoffman et al. 2010; Pagarete et al. 2005; Teske et al. 2007). Morphological variability can lead to species misidentification with serious implications for monitoring activities and conservation policies in MPAs. The aim of the present study was to investigate the morphometric measures able to discriminate between the two most common Mediterranean species: *Patella rustica* and *Patella caerulea*.

Results

The two investigated limpet species had similar sizes, however, some morphometric ratios were able to discriminate the two species as shown by the PCA ordination plot (Fig. 3a). In particular the ratio Height/Maximum Diameter was the most effective in discriminating the two species at all sites (PERMANOVA $P<0.01$, Fig. 3b). Moreover, the two species significantly differ in the mean Circularity at all sites (pairwise $P<0.01$), except in CAL; in the ratio Weight/Maximum diameter, except in ARG and CAPLAR, in the mean shell Solidity, except at CAL and FAROPAL (Fig. 3c), and in the ratio Height/Area, except in LEV and FAROPAL (Fig. 3d).

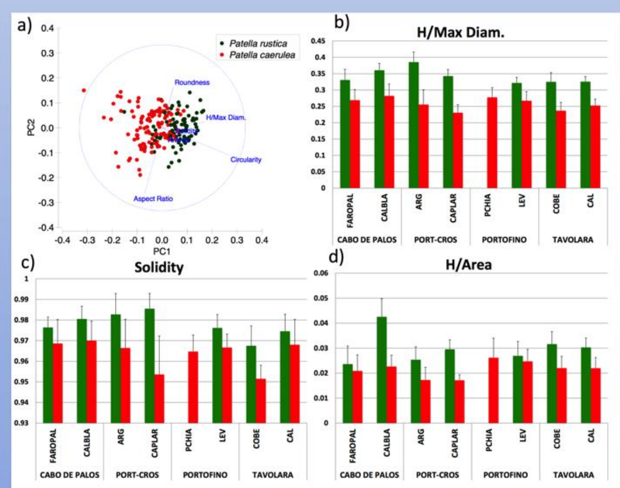


Fig. 3. Main results: a) PCA plot; b) H/Max Diameter; c) Solidity; d) Height/Area (mean + SD). FAROPAL = Faro Cabo de Palos, CALBLA = Calblanque, ARG = Argentiere, CAPLAR = Cap Lardier, PCHIA = Punta Chiappa, LEV = Sestri Levante, COBE = Corallina Beach, CAL = La Calleta).

Conclusion

Despite the morphological differences among species appeared consistent, a morphological variability among sites was found. H/Max Diam. and Circularity were the most effective in distinguishing the two species. Selected morphometric measures may be an useful tool for species identification when when genetic tests are not a feasible alternative.

Acknowledgments

We wish to thank Adriana Villamor for her advice in the laboratory and to Ramón Hernández, José Chartón, Jose Manuel Perñiguez, Ubaldo Pantaleo and Marco Palma for their collaboration in the sampling. Research funded by the Training Network for Monitoring Mediterranean Marine Protected Areas (MMMPA) under the European Community's 7th Framework Programme (Grant Agreement no.: 290056).

Fig. 1. Map of study sites.

Material & Methods

Two closely related limpets, *Patella rustica* (n=86) and *P. caerulea* (n=114), were collected in 8 sites in the western Mediterranean MPAs of Cabo de Palos, Port-Cros, Tavolara and Portofino (except for *P. rustica* in Punta Chiappa that was barely present; Fig. 1). To avoid species misidentification, a portion of mitochondrial COI region for all the individuals were sequenced (Fig. 2). Morphometric measures were obtained by analytical balance (Weight), digital calliper (Height) and image analysis (Max Diameter, Aspect Ratio of the shell's fitted ellipse, Circularity - a measure of how the shape fits the circle, Roundness - invers of Aspect-ratio, Solidity - a measure of how "ruffled" the borders are), using ImageJ. Main allometric ratios were calculated (Height/Area, Height/Maximum Diameter, Weight/Maximum Diameter) and differences of the mean values between species, at each sites, were investigated with the Principal Component Analysis (PCA; Pearson, 1901) and tested by permutational multivariate analysis of variance (PERMANOVA; Anderson and ter Braak, 2003).

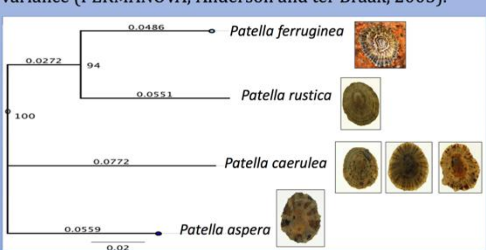


Fig. 2. Rooted-Neighbour joining tree of the COI from *Patella rustica* and *P. caerulea*, showing their genetic distinctness. *P. aspera* was used as an out-group. Numbers in the branches represent the substitutions per site and numbers in the nodes represent the bootstrap values.

References

Anderson, M.J., ter Braak, C.J.F. (2003). Permutation tests for multi-factorial analysis of variance. J. Stat. Comput. Sim. 73(2), 85-113.

Hoffman, J. I., Peck, L. S., Hillyard, G., Zieritz, A., & Clark, M. S. (2010). No evidence for genetic differentiation between Antarctic limpet *Nacella concinna* morphotypes. *Marine Biology*, 157(4), 765-778

Pearson, K. (1901). LIII. On lines and planes of closest fit to systems of points in space. Philos. Mag. Series 6 2(11), 559-572.

Pagarete, A.J., Costa, M.J., & Brito, C. (2005). Genetic conspecifity in two habitat morphs of the limpet *Patella candei gomesii* Drouët (Mollusca: Gastropoda: Patellidae) reinforces limpet morphological plasticity. *J. Young Invest.*, 12(5).

Teske, P.R., Barker, N.P., & McQuaid, C.D. (2007). Lack of genetic differentiation among four sympatric southeast African intertidal limpets (Siphonariidae): phenotypic plasticity in a single species? *J. Mollus. Stud.*, 73(3), 223-228.

Conference: MMMPA, Ancona, Italy. 15 - 17 October, 2015

Guidelines on genetic connectivity as a tool for assessing the effectiveness of Marine Protected Areas (abstract)

P. Marti-Puig[1,] A. Calò[2], F. Costantini[1], A. Villamor[1], M. Abbiati[1], M. Ponti[1], J. A. García-Charton[2]

[1] BiGeA & CIRSA, Università di Bologna, Ravenna, Italy. * E-mail: patypuig@gmail.com

[2] Department of Ecology and Hydrology, Universidad de Murcia, Murcia, Spain.

The design and management of Marine Protected Areas (MPAs) and MPA networks should take into account the spatial distribution patterns and connectivity among populations of the target species, as a key element in biological conservation. Connectivity is the exchange of individuals among populations through the passive transport and/or active movement of individuals at whatever life stage. Well-connected and highly diverse populations are more resilient to natural and anthropogenic environmental impacts. In the context of MPA monitoring, genetic analyses are considered a powerful tool for assessing population diversity and connectivity patterns at different temporal and spatial scales. In this poster presentation the guidelines to apply genetic analyses as a monitoring tool for MPAs are presented. Two case studies in which genetics tools were used to assess connectivity patterns between protected and unprotected areas in the Western Mediterranean Sea were provided. In these case studies, two widely distributed intertidal limpets, *Patella rustica* and *Patella caerulea*, and a commercially renowned coastal fish, the saddled sea bream *Oblada melanura*, were considered. The results of these studies provide MPA managers with good examples on how to apply these guidelines and obtain the information needed to address specific species conservation issues.

Genetic connectivity as a tool for assessing the effectiveness of Marine Protected Areas. MMMPA final conference. MMMPA final conference (poster)

MMMPA

GENETIC CONNECTIVITY AS A TOOL FOR ASSESSING THE EFFECTIVENESS OF MARINE PROTECTED AREAS

P. Marti-Puig[1]§*, A. Calò[2]#*, F. Costantini[1], A. Villamor[1], M. Abbiati[1], M. Ponti[1], J. A. García-Chartón[2]

[1] Dipartimento di Scienze Biologiche, Geologiche ed Ambientali & Centro Interdisciplinare di Ricerca per le Scienze Ambientali, University of Bologna, Ravenna, Italy
[2] Department of Ecology and Hydrology, University of Murcia, Murcia, Spain

§ patypuig@gmail.com

* These authors contributed equally to this work

antonio.calo@um.es

Why and how to monitor connectivity

Connectivity is the exchange of individuals among populations through the passive transport and/or active movement of individuals at whatever life stage (i.e. gametes, larvae, juveniles, sub-adults and adults) (Cowen and Sponaugle, 2009).

Connectivity is a fundamental aspect to consider when evaluating the status of existing MPAs and their ability to participate in an effective network, since well-connected and highly diverse populations are more resilient to environmental impacts and less likely to face local extinctions (Planes S, 2009).

The investigation of connectivity patterns can provide information on:

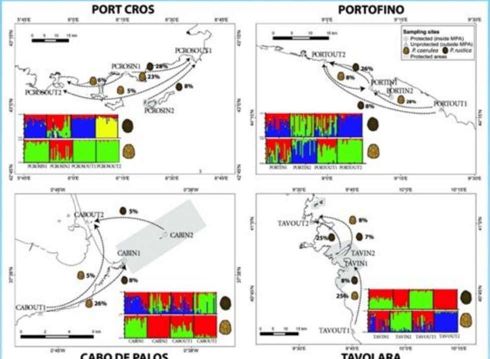
(1) portion of reproduction output of protected populations retained within MPA borders

(2) amount of individuals exported from protected populations toward unprotected areas

(3) strength and direction of connections (both genetic and demographic) between a MPA and the other MPAs within a network

This information can help managers to assess the status of their MPAs, and to address specific management issues in order to improve and/or maintain MPA health and effectiveness

In the context of MPA monitoring, genetic analyses are considered a powerful tool for assessing connectivity at different temporal and spatial scales as they are non-lethal (Calò et al., 2013, Marti-Puig et al., 2013) and can be used to investigate diversity and connectivity patterns in a huge variety of marine organisms with standard protocols equally valid for all animal or plant taxa.

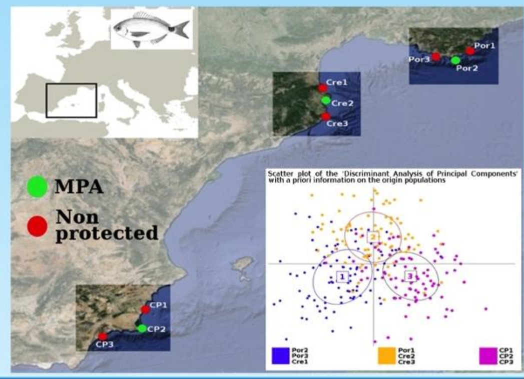


A case study on invertebrates: the limpets

The efficiency of four western Mediterranean MPAs was assessed comparing the genetic variability of two species of limpets (Patella caerulea and Patella rustica) in protected and non-protected sites. Mitochondrial cytochrome oxidase c subunit 1 region was used for evaluating the connectivity among MPAs whereas the more variable microsatellite markers were used for evaluating connectivity patterns within and around each MPA. Genetic diversity differed among protected and non-protected sites for both species, with higher extent for P. caerulea. However, this "MPA effect" was not consistent in all locations. At geographic level, both species showed high genetic connectivity between Port-Cros, Tavolara and Portofino locations (including MPA and surrounding sites) with slight significant genetic differentiation with the most southwestern location (Cabo de Palos). At local MPA level, no protection effect was observed in genetic diversity. Moreover within each MPA, different patterns of genetic structure and connectivity were observed depending on species and local environmental features. The results of this study suggest that multi-species and multi-scale management approaches are needed to evaluate the efficiency of MPAs.

A case study on fishes: the saddled seabream

The genetic structure of the saddled seabream (Oblada melanura) and the level of genetic connectivity between protected and unprotected populations was investigated using a set of 11 microsatellite loci, considering three MPAs of the West Mediterranean Sea. All values of population differentiation were non-significant after Bonferroni correction, indicating that, at local spatial scale (50-100 km), protected populations were in general well connected with non-protected ones. On the other hand, at the regional scale (500-1000 km), statistical analyses revealed the presence of a subtle population structure that reflects the main oceanographic features of the study area. These results may have important implications for the conservation biology and fisheries management of saddled sea bream like other coastal fish, as spatial variability in connectivity patterns may promote long-term stability of fish populations. From this perspective, multi-scale patterns of genetic connectivity should be taken into account when future MPA will be established in the West Mediterranean Sea, implementing the existing network.



Remarks

MPA design and monitoring based on connectivity assessment should take into account:

1) The knowledge of the biology and ecology of the model species, including:
   - Life history traits, habitat preferences and behaviour
   - Larval dispersal capability and movement characteristics
   - Genetic background

2) The local conditions of the MPA of interest or the possible MPA network, including:
   - Information on oceanic patterns
   - Information of the habitat characteristics

References

- Calò, A. et al, 2013. A review of methods to assess connectivity and dispersal between fish populations in the Mediterranean Sea. Adv. Oceanogr. Limnol. 4, 150-175
- Cowen, R.K. and Sponaugle, S., 2009. Larval dispersal and marine population connectivity. Annual Review of Marine Science 1, 443-466
- Marti-Puig, P. et al, 2013. Patterns of genetic connectivity in invertebrates of temperate MPA networks. Adv. Oceanogr. Limnol. 4, 138-149
- Planes S et al, 2009. Larval dispersal connects fish populations in a network of marine protected areas. PNAS,105, 5693-5697

This research was supported by the European project 'Initial Training Network for Monitoring Mediterranean Marine Protected Areas' (MMMPA: FP7-PEOPLE-2011-ITN) [grant number 290056].

References

Abdulla, A., Gomei, M., Hyrenbach, D., Notarbartolo-di-Sciara, G., Agardy, T., 2009. Challenges facing a network of representative marine protected areas in the Mediterranean: prioritizing the protection of underrepresented habitats. ICES Journal of Marine Science: Journal du Conseil 66, 22-28.

Agardy, M.T., 1994. Advances in marine conservation: the role of marine protected areas. Trends in Ecology & Evolution 9, 267-270.

Airoldi, L., Beck, M.W., 2007. Loss, status and trends for coastal marine habitats of Europe. Oceanogr Mar Biol 45, 345-405.

Almany, G.R., Connolly, S.R., Heath, D.D., Hogan, J.D., Jones, G.P., McCook, L.J., Mills, M., Pressey, R.L., Williamson, D.H., 2009. Connectivity, biodiversity conservation and the design of marine reserve networks for coral reefs. Coral Reefs 28, 339-351.

Andrello, M., Jacobi, M.N., Manel, S., Thuiller, W., Mouillot, D., 2015. Extending networks of protected areas to optimize connectivity and population growth rate. Ecography 38, 273-282.

Arrontes, J., Arenas, F., Fernandez, C., Rico, J., Oliveros, J., Martinez, B., Viejo, R., Alvarez, D., 2004. Effect of grazing by limpets on mid-shore species assemblages in northern Spain. Marine Ecology Progress Series 277, 117-133.

Babcock, R., Shears, N., Alcala, A., Barrett, N., Edgar, G., Lafferty, K., McClanahan, T., Russ, G., 2010. Decadal trends in marine reserves reveal differential rates of change in direct and indirect effects. Proceedings of the National Academy of Sciences 107, 18256-18261.

Bacci, G., Sella, G., 1970. Correlations between characters and environmental conditions in Patella of the coerulea group. Pubblicazioni della Stazione Zoologica di Napoli 38, 1-17.

Bannister, J., 1975. Shell parameters in relation to zonation in Mediterranean limpets. Marine biology 31, 63-67.

Baskett, M.L., Micheli, F., Levin, S.A., 2007. Designing marine reserves for interacting species: insights from theory. Biological Conservation 137, 163-179.

Basterretxea, G., Jordi, A., CATALÁN, I.A., Sabatés, A., 2012. Model- based assessment of local- scale fish larval connectivity in a network of marine protected areas. Fisheries Oceanography 21, 291-306.

Becker, B.J., Levin, L.A., Fodrie, F.J., McMillan, P.A., 2007. Complex larval connectivity patterns among marine invertebrate populations. Proceedings of the National Academy of Sciences 104, 3267-3272.

Beger, M., Selkoe, K.A., Treml, E., Barber, P.H., von der Heyden, S., Crandall, E.D., Toonen, R.J., Riginos, C., 2014. Evolving coral reef conservation with genetic information. Bulletin of Marine Science 90, 159-185.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N., Bonhomme, F., 2004. GENETIX 4.05, Population genetics software for Windows TM. Université de Montpellier II. Montpellier.

Bell, J.J., 2008a. Connectivity between island Marine Protected Areas and the mainland. Biological Conservation 141, 2807-2820.

Bell, J.J., 2008b. Similarity in connectivity patterns for two gastropod species lacking pelagic larvae. Marine Ecology Progress Series 357, 185-194.

Bell, J.J., 2012. High connectivity between sea lough populations of a planktonic larval disperser with the adjacent open coast. Marine Ecology-an Evolutionary Perspective 33, 516-521.

Bellwood, D., Hughes, T., Folke, C., Nyström, M., 2004. Confronting the coral reef crisis. Nature 429, 827-833.

Benedetti-Cecchi, L., 2001. Variability in abundance of algae and invertebrates at different spatial scales on rocky sea shores. Marine Ecology Progress Series 215, e92.

Benedetti-Cecchi, L., Maggi, E., Bertocci, I., Vaselli, S., Micheli, F., Osio, G., Cinelli, F., 2003. Variation in rocky shore assemblages in the northwestern Mediterranean: contrasts between islands and the mainland. Journal of Experimental Marine Biology and Ecology 293, 193-215.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological), 289-300.

Bernhardt, J.R., Leslie, H.M., 2013. Resilience to climate change in coastal marine ecosystems. Marine Science 5.

Berumen, M.L., Almany, G.R., Planes, S., Jones, G.P., Saenz-Agudelo, P., Thorrold, S.R., 2012. Persistence of self-recruitment and patterns of larval connectivity in a marine protected area network. Ecology and Evolution 2, 444-452.

Bianchi, C., Morri, C., 2000. Marine biodiversity of the Mediterranean Sea: situation, problems and prospects for future research. Marine Pollution Bulletin 40, 367-376.

Bookstein, F.L., 1997. Landmark methods for forms without landmarks: morphometrics of group differences in outline shape. Medical image analysis 1, 225-243.

Bosman, A., Hockey, P., Siegfried, W., 1987. The influence of coastal upwelling on the functional structure of rocky intertidal communities. Oecologia 72, 226-232.

Bouffard, J., Pascual, A., Ruiz, S., Faugère, Y., Tintoré, J., 2010. Coastal and mesoscale dynamics characterization using altimetry and gliders: A case study in the Balearic Sea. Journal of Geophysical Research: Oceans 115.

Bouzat, J.L., 2010. Conservation genetics of population bottlenecks: the role of chance, selection, and history. Conservation Genetics 11, 463-478.

Burgos-Rubio, V., De la Rosa, J., Altamirano, M., Espinosa, F., 2015. The role of patellid limpets as omnivorous grazers: a new insight into intertidal ecology. Marine Biology 162, 2093-2106.

Cadrin, S.X., Friedland, K.D., 1999. The utility of image processing techniques for morphometric analysis and stock identification. Fisheries Research 43, 129-139.

Calò, A., Félix-Hackradt, F.C., Garcia, J., Hackradt, C.W., Rocklin, D., Treviño Otón, J., Charton, J.A.G., 2013. A review of methods to assess connectivity and dispersal between fish populations in the Mediterranean Sea. Advances in Oceanography and Limnology 4, 150-175.

Ceccherelli, G., Pinna, S., Navone, A., Sechi, N., 2011. Influence of Geographical Siting and Protection on Rocky-Shore Gastropod Distribution at a Western Mediterranean Marine Protected Area. Journal of Coastal Research 27, 882-889.

Chapuis, M.-P., Estoup, A., 2007. Microsatellite null alleles and estimation of population differentiation. Molecular biology and evolution 24, 621-631.
Charton, J.G., Williams, I., Ruzafa, A.P., Milazzo, M., Chemello, R., Marcos, C., Kitsos, M., Koukouras, A., Riggio, S., 2000. Evaluating the ecological effects of Mediterranean marine protected areas: habitat, scale and the natural variability of ecosystems. Environmental conservation 27, 159-178.
Christianen, M.J., Herman, P.M., Bouma, T.J., Lamers, L.P., van Katwijk, M.M., van der Heide, T., Mumby, P.J., Silliman, B.R., Engelhard, S.L., van de Kerk, M., 2014. Habitat collapse due to overgrazing threatens turtle conservation in marine protected areas. Proceedings of the Royal Society of London B: Biological Sciences 281, 20132890.
Claudet, J., Osenberg, C.W., Benedetti-Cecchi, L., Domenici, P., Garcia-Charton, J.A., Perez-Ruzafa, A., Badalamenti, F., Bayle-Sempere, J., Brito, A., Bulleri, F., Culioli, J.M., Dimech, M., Falcon, J.M., Guala, I., Milazzo, M., Sanchez-Meca, J., Somerfield, P.J., Stobart, B., Vandeperre, F., Valle, C., Planes, S., 2008. Marine reserves: size and age do matter. Ecol Lett 11, 481-489.
Claudet, J., Osenberg, C.W., Domenici, P., Badalamenti, F., Milazzo, M., Falcon, J.M., Bertocci, I., Benedetti-Cecchi, L., Garcia-Charton, J.A., Goni, R., Borg, J.A., Forcada, A., De Lucia, G.A., Perez-Ruzafa, A., Afonso, P., Brito, A., Guala, I., Le Direach, L., Sanchez-Jerez, P., Somerfield, P.J., Planes, S., 2010. Marine reserves: fish life history and ecological traits matter. Ecol Appl 20, 830-839.
Coleman, M.A., Chambers, J., Knott, N.A., Malcolm, H.A., Harasti, D., Jordan, A., Kelaher, B.P., 2011. Connectivity within and among a Network of Temperate Marine Reserves. PloS one 6, e20168.
Coleman, R.A., Underwood, A.J., Benedetti-Cecchi, L., Åberg, P., Arenas, F., Arrontes, J., Castro, J., Hartnoll, R.G., Jenkins, S.R., Paula, J., 2006. A continental scale evaluation of the role of limpet grazing on rocky shores. Oecologia 147, 556-564.
Coll, M., Piroddi, C., Steenbeek, J., Kaschner, K., Lasram, F.B., Aguzzi, J., Ballesteros, E., Bianchi, C.N., Corbera, J., Dailianis, T., Danovaro, R., Estrada, M., Froglia, C., Galil, B.S., Gasol, J.M., Gertwagen, R., Gil, J., Guilhaumon, F., Kesner-Reyes, K., Kitsos, M.S., Koukouras, A., Lampadariou, N., Laxamana, E., de la Cuadra, C.M.L.F., Lotze, H.K., Martin, D., Mouillot, D., Oro, D., Raicevich, S., Rius-Barile, J., Saiz-Salinas, J.I., San Vicente, C., Somot, S., Templado, J., Turon, X., Vafidis, D., Villanueva, R., Voultsiadou, E., 2010. The Biodiversity of the Mediterranean Sea: Estimates, Patterns, and Threats. Plos One 5.
Coma, R., Linares, C., Ribes, M., Diaz, D., Garrabou, J., Ballesteros, E., 2006. Consequences of a mass mortality in populations of *Eunicella singularis* (Cnidaria: Octorallia) in Menorca (NW Mediterranean).
Cornuet, J.M., Luikart, G., 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144, 2001-2014.
Côrte-Real, H., Macaulay, V., Richards, M.B., Hariti, G., Issad, M., CAMBON-THOMSEN, A., Papiha, S., Bertranpetit, J., Sykes, B., 1996. Genetic diversity in the Iberian Peninsula determined from mitochondrial sequence analysis. Annals of human genetics 60, 331-350.
Cossu, P., Dedola, G.L., Scarpa, F., Sanna, D., Lai, T., Maltagliati, F., Curini-Galletti, M., Casu, M., 2015. Patterns of spatial genetic variation in Patella

ulyssiponensis: insights from the western Mediterranean marine ecoregion. Hydrobiologia 755, 39-55.

Costa, M., Richter, A., Koivunen, V., 2010. Unified array manifold decomposition based on spherical harmonics and 2-D Fourier basis. Signal Processing, IEEE Transactions on 58, 4634-4645.

Costantini, F., Carlesi, L., Abbiati, M., 2013. Quantifying spatial genetic structuring in mesophotic populations of the precious coral Corallium rubrum. PloS one 8, e61546.

Costantini, F., Fauvelot, C., Abbiati, M., 2007a. Fine-scale genetic structuring in Corallium rubrum: evidence of inbreeding and limited effective larval dispersal. Marine Ecology Progress Series 340, 109-119.

Cowen, R.K., Lwiza, K.M., Sponaugle, S., Paris, C.B., Olson, D.B., 2000. Connectivity of marine populations: open or closed? Science 287, 857-859.

Cowen, R.K., Sponaugle, S., 2009. Larval dispersal and marine population connectivity. Annual Review of Marine Science 1, 443-466.

Crampton, J., 1995. Elliptic Fourier shape analysis of fossil bivalves: some practical considerations. Lethaia 28, 179-186.

Crooks, K.R., 2006. Connectivity conservation. Cambridge University Press.

Crowder, L.B., Lyman, S., Figueira, W., Priddy, J., 2000. Source-sink population dynamics and the problem of siting marine reserves. Bulletin of Marine Science 66, 799-820.

Csencsics, D., Brodbeck, S., Holderegger, R., 2010. Cost-effective, species-specific microsatellite development for the endangered dwarf bulrush (Typha minima) using next-generation sequencing technology. Journal of Heredity, esq069.

Davenport, J., Davenport, J.L., 2006. The impact of tourism and personal leisure transport on coastal environments: a review. Estuarine, Coastal and Shelf Science 67, 280-292.

de Aranzamendi, M.C., Martínez, J.J., Sahade, R., 2010. Shape differentiation and characterization in the two morphotypes of the Antarctic limpet *Nacella concinna* using Elliptic Fourier analysis of shells. Polar biology 33, 1163-1170.

Dempster, A.P., Laird, N.M., Rubin, D.B., 1977. Maximum likelihood from incomplete data via the EM algorithm. Journal of the royal statistical society. Series B (methodological), 1-38.

Di Franco, A., Coppini, G., Pujolar, J.M., De Leo, G.A., Gatto, M., Lyubartsev, V., Melia, P., Zane, L., Guidetti, P., 2012. Assessing Dispersal Patterns of Fish Propagules from an Effective Mediterranean Marine Protected Area. Plos One 7.

Dodd, J., 1957. Artificial fertilization, larval development and metamorphosis in *Patella vulgata* L. and *Patella caerulea L*. Pubblicazioni della Stazione Zoologica di Napoli 29, 172-185.

Doglioli, A.M., Griffa, A., Magaldi, M.G., 2004. Numerical study of a coastal current on a steep slope in presence of a cape: The case of the Promontorio di Portofino. Journal of Geophysical Research: Oceans 109.

Drew, J.A., Barber, P.H., 2012. Comparative Phylogeography in Fijian Coral Reef Fishes: A Multi-Taxa Approach towards Marine Reserve Design. Plos One 7.

Dufresne, F., Bourget, E., Bernatchez, L., 2002. Differential patterns of spatial divergence in microsatellite and allozyme alleles: further evidence for

locus- specific selection in the acorn barnacle, *Semibalanus balanoides*? Molecular Ecology 11, 113-123.

Dufresne, F., Parent, M., Bernatchez, L., 1999. Isolation and characterization of microsatellite markers in the acorn barnacle *Semibalanus balanoides*. Mol Ecol 8, 1558-1559.

Earl, D.A., BM, v., 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation genetics resources 4, 359-361.

Edgar, G.J., Stuart-Smith, R.D., Willis, T.J., Kininmonth, S., Baker, S.C., Banks, S., Barrett, N.S., Becerro, M.A., Bernard, A.T., Berkhout, J., 2014. Global conservation outcomes depend on marine protected areas with five key features. Nature 506, 216-220.

Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular ecology 14, 2611-2620.

Evans, R.G., 1953. Studies on the biology of British limpets—the genus Patella on the south coast of England, Proceedings of the Zoological Society of London. Wiley Online Library, pp. 357-376.

Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131, 479-491.

Falush, D., Stephens, M., Pritchard, J.K., 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164, 1567-1587.

Fauvelot, C., Bertozzi, F., Costantini, F., Airoldi, L., Abbiati, M., 2009. Lower genetic diversity in the limpet *Patella caerulea* on urban coastal structures compared to natural rocky habitats. Marine Biology 156, 2313-2323.

Fauvelot, C., Costantini, F., Virgilio, M., Abbiati, M., 2012. Do artificial structures alter marine invertebrate genetic makeup? Marine biology 159, 2797-2807.

Fenberg, P.B., Roy, K., 2012. Anthropogenic harvesting pressure and changes in life history: insights from a rocky intertidal limpet. The American Naturalist 180, 200-210.

Foster, N.L., Paris, C.B., KOOL, J.T., Baums, I.B., Stevens, J.R., Sanchez, J.A., Bastidas, C., Agudelo, C., Bush, P., Day, O., 2012. Connectivity of Caribbean coral populations: complementary insights from empirical and modelled gene flow. Mol Ecol 21, 1143-1157.

Frankham, R., 2005a. Genetics and extinction. Biological conservation 126, 131-140.

Freeman, H., 1974. Computer processing of line-drawing images. ACM Computing Surveys (CSUR) 6, 57-97.

Friedland, K., 1996. Analyses of calcified structures–Fourier shape analysis. ICES CM 1000, 1.

Gaines, S.D., White, C., Carr, M.H., Palumbi, S.R., 2010. Designing marine reserve networks for both conservation and fisheries management. Proceedings of the National Academy of Sciences 107, 18286-18293.

Galarza, J.A., Carreras-Carbonell, J., Macpherson, E., Pascual, M., Roques, S., Turner, G.F., Rico, C., 2009. The influence of oceanographic fronts and early-life-history traits on connectivity among littoral fish species. Proceedings of the National Academy of Sciences 106, 1473-1478.

Gleick, P.H., Adams, R., Amasino, R., Anders, E., Anderson, D., Anderson, W., Anselin, L., Arroyo, M., Asfaw, B., Ayala, F., 2010. Climate change and the integrity of science. Science 328, 689-690.
González-Wangüemert, M., Aydin, M., Conand, C., 2014. Assessment of sea cucumber populations from the Aegean Sea (Turkey): First insights to sustainable management of new fisheries. Ocean & Coastal Management 92, 87-94.
González-Wevar, C., Nakano, T., Cañete, J., Poulin, E., 2011. Concerted genetic, morphological and ecological diversification in *Nacella* limpets in the Magellanic Province. Molecular Ecology 20, 1936-1951.
Green, A.L., Maypa, A.P., Almany, G.R., Rhodes, K.L., Weeks, R., Abesamis, R.A., Gleason, M.G., Mumby, P.J., White, A.T., 2014. Larval dispersal and movement patterns of coral reef fishes, and implications for marine reserve network design. Biological Reviews.
Guerra, M.T., Gaudencio, M.J., 1986. Aspects of the ecology of *Patella* spp. on the Portuguese coast. Hydrobiologia 142, 57-69.
Guerra-Garcıa, J.M., Corzo, J., Espinosa, F., Garcıa-Gomez, J.C., 2004. Assessing habitat use of the endangered marine mollusc *Patella ferruginea* (Gastropoda, Patellidae) in northern Africa: preliminary results and implications for conservation. Biological conservation 116, 319-326.
Guidetti, P., Notarbartolo- Di- Sciara, G., Agardy, T., 2013. Integrating pelagic and coastal MPAs into large- scale ecosystem- wide management. Aquatic Conservation: Marine and Freshwater Ecosystems 23, 179-182.
Halpern, B.S., Walbridge, S., Selkoe, K.A., Kappel, C.V., Micheli, F., D'Agrosa, C., Bruno, J.F., Casey, K.S., Ebert, C., Fox, H.E., 2008. A global map of human impact on marine ecosystems. Science 319, 948-952.
Hastings, A., Botsford, L.W., 2006. Persistence of spatial populations depends on returning home. Proceedings of the National Academy of Sciences 103, 6067-6072.
Hebert, P.D.N., Cywinska, A., Ball, S.L., deWaard, J.R., 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London Series B, 313–321.
Hedgecock, D., Barber, P.H., Edmands, S., 2007. Genetic approaches to measuring connectivity. Oceanography-Washington DC-Oceanography society 20, 70.
Hellberg, M.E., 1996. Dependence of gene flow on geographic distance in two solitary corals with different larval dispersal capabilities. Evolution, 1167-1175.
Hellberg, M.E., Burton, R.S., Neigel, J.E., Palumbi, S.R., 2002. Genetic assessment of connectivity among marine populations. Bulletin of marine science 70, 273-290.
Hiraoka, Y., Kuramoto, N., Center, F.T.B., 2004. Identification of Rhus succedanea L. Cultivars Using Elliptic Fourier Descriptors Based on Fruit Shape. Silvae Genetica 53, 221-226.
Hoffmann, A.A., Sgrò, C.M., 2011. Climate change and evolutionary adaptation. Nature 470, 479-485.
Hubisz, M.J., Falush, D., Stephens, M., Pritchard, J.K., 2009. Inferring weak population structure with the assistance of sample group information. Molecular ecology resources 9, 1322-1332.
Hughes, T.P., Baird, A.H., Bellwood, D.R., Card, M., Connolly, S.R., Folke, C., Grosberg, R., Hoegh-Guldberg, O., Jackson, J., Kleypas, J., 2003. Climate

change, human impacts, and the resilience of coral reefs. Science 301, 929-933.
Hutchings, J.A., Reynolds, J.D., 2004. Marine fish population collapses: consequences for recovery and extinction risk. BioScience 54, 297-309.
Iwata, H., Ukai, Y., 2002. SHAPE: a computer program package for quantitative evaluation of biological shapes based on elliptic Fourier descriptors. Journal of Heredity 93, 384-385.
Jaquiéry, J., Broquet, T., Hirzel, A., Yearsley, J., Perrin, N., 2011. Inferring landscape effects on dispersal from genetic distances: how far can we go? Mol Ecol 20, 692-705.
Jones, G.P., Almany, G.R., Russ, G.R., Sale, P.F., Steneck, R.S., van Oppen, M.J.H., Willis, B.L., 2009. Larval retention and connectivity among populations of corals and reef fishes: history, advances and challenges. Coral Reefs 28, 307-325.
Jost, L., 2008. GST and its relatives do not measure differentiation. Molecular Ecology 17, 4015-4026.
Kaesler, R.L., Waters, J.A., 1972. Fourier analysis of the ostracode margin. Geological Society of America Bulletin 83, 1169-1178.
Kalinowski, S.T., 2004. Counting alleles with rarefaction: private alleles and hierarchical sampling designs. Conservation Genetics 5, 539-543.
Kaplan, D.M., Botsford, L.W., O'Farrell, M.R., Gaines, S.D., Jorgensen, S., 2009. Model-based assessment of persistence in proposed marine protected area designs. Ecological Applications 19, 433-448.
Kaunda-Arara, B., Rose, G.A., 2004. Effects of marine reef National Parks on fishery CPUE in coastal Kenya. Biological Conservation 118, 1-13.
Kelleher, G., 1996. A global representative system of marine protected areas. Ocean & coastal management 32, 123-126.
Kelly, R.P., Palumbi, S.R., 2010. Genetic Structure Among 50 Species of the Northeastern Pacific Rocky Intertidal Community. Plos One 5.
Kennish, M.J., 2002. Environmental threats and environmental future of estuaries. Environmental conservation 29, 78-107.
Keough, M.J., Quinn, G., 1998. Effects of periodic disturbances from trampling onrocky intertidal algal beds. Ecological Applications 8, 141-161.
Kool, J.T., Moilanen, A., Treml, E.A., 2013. Population connectivity: recent advances and new perspectives. Landscape Ecology 28, 165-185.
Kopelman, N.M., Mayzel, J., Jakobsson, M., Rosenberg, N.A., Mayrose, I., 2015. Clumpak: a program for identifying clustering modes and packaging population structure inferences across K. Molecular ecology resources 15, 1179-1191.
Kotsakiozi, P., Rigal, F., Valakos, E.D., Parmakelis, A., 2013. Disentangling the effects of intraspecies variability, phylogeny, space, and climate on the evolution of shell morphology in endemic Greek land snails of the genus Codringtonia. Biological Journal of the Linnean Society 110, 796-813.
Kress, W.J., Erickson, D.L., 2008. DNA barcodes: genes, genomics, and bioinformatics. Proceedings of the National Academy of Sciences 105, 2761-2762.
Kritzer, J.P., Sale, P.F., 2004. Metapopulation ecology in the sea: from Levins' model to marine ecology and fisheries science. Fish and Fisheries 5, 131-140.
Kuhl, F.P., Giardina, C.R., 1982. Elliptic Fourier features of a closed contour. Computer graphics and image processing 18, 236-258.

Kyle, C., Boulding, E., 2000. Comparative population genetic structure of marine gastropods (*Littorina spp.*) with and without pelagic larval dispersal. Marine Biology 137, 835-845.

Lotze, H.K., Coll, M., Magera, A.M., Ward-Paige, C., Airoldi, L., 2011. Recovery of marine animal populations and ecosystems. Trends in Ecology & Evolution 26, 595-605.

Lowe, W.H., Allendorf, F.W., 2010. What can genetics tell us about population connectivity? Molecular Ecology 19, 3038-3051.

Lubchenco, J., Palumbi, S.R., Gaines, S.D., Andelman, S., 2003. Plugging a hole in the ocean: the emerging science of marine reserves 1. Ecological applications 13, 3-7.

MacLeod, N., Ortiz, N., Fefferman, N., Clyde, W., Schulter, C., MacLean, J., 2000. Phenotypic response of foraminifera to episodes of global environmental change. Biotic response to global environmental change: the last 145, 51-78.

Marshall, D., Monro, K., Bode, M., Keough, M., Swearer, S., 2010. Phenotype–environment mismatches reduce connectivity in the sea. Ecol Lett 13, 128-140.

Marti-Puig, P., Calò, A., Costantini, F., Villamor, A., Abbiati, M., Ponti, M., García-Charton, J.A., 2016. Genetic connectivity and diversity as a tool to assess the effectiveness of Marine Protected Areas. in: MMMPA Supervisory Board (Ed.), Monitoring Mediterranean Marine Protected Areas: A set of guidelines to support the development of management plans. Deliverable of the MMMPA European project (FP7-PEOPLE-2011-ITN g.a. no.: 290056), Ancona, pp. 81-87.

Marti-Puig, P., Costantini, F., Rugiu, L., Ponti, M., Abbiati, M., 2013. Patterns of genetic connectivity in invertebrates of temperate MPA networks. Advances in Oceanography and Limnology 4, 138-149.

Mauro, A., Arculeo, M., Parrinello, N., 2003. Morphological and molecular tools in identifying the Mediterranean limpets *Patella caerulea*, *Patella aspera* and *Patella rustica*. Journal of Experimental Marine Biology and Ecology 295, 131-143.

McInerney, C.E., Allcock, A.L., Johnson, M.P., Prodohl, P.A., 2009a. Characterization of polymorphic microsatellites for the rough periwinkle gastropod, *Littorina saxatilis* (Olivi, 1792) and their cross-amplification in four congeners. Conservation Genetics 10, 1989-1992.

McInerney, C.E., Allcock, A.L., Johnson, M.P., Prodohl, P.A., 2009b. Understanding marine reserve function in a seascape genetics context: *Nucella lapillus* in Strangford Lough (Northern Ireland) as an example. Aquatic Biology 7, 45-58.

Meirmans, P.G., 2014. Non-convergence in Bayesian estimation of migration rates. Molecular Ecology Resources 14, 726-733.

Menconi, M., Benedetti-Cecchi, L., Cinelli, F., 1999. Spatial and temporal variability in the distribution of algae and invertebrates on rocky shores in the northwest Mediterranean. Journal of Experimental Marine Biology and Ecology 233, 1-23.

Miller, K.J., Ayre, D.J., 2008. Protection of genetic diversity and maintenance of connectivity among reef corals within marine protected areas. Conservation Biology 22, 1245-1254.

Mokhtar-Jamai, K., Pascual, M., Ledoux, J.B., Coma, R., Feral, J.P., Garrabou, J., Aurelle, D., 2011. From global to local genetic structuring in the red

gorgonian *Paramuricea clavata*: the interplay between oceanographic conditions and limited larval dispersal. Molecular Ecology 20, 3291-3305.
Moore, H.B., 1934. The relation of shell growth to environment in Patella yulgata. Journal of Molluscan Studies 21, 217-222.
Munguía-Vega, A., Sáenz-Arroyo, A., Greenley, A.P., Espinoza-Montes, J.A., Palumbi, S.R., Rossetto, M., Micheli, F., 2015. Marine reserves help preserve genetic diversity after impacts derived from climate variability: Lessons from the pink abalone in Baja California. Global Ecology and Conservation 4, 264-276.
Nei, M., 1987. Molecular evolutionary genetics. Columbia university press.
Nei, M., Li, W.-H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences 76, 5269-5273.
Padilla, D.K., Savedo, M.M., , 2013. A systematic review of phenotypic plasticity in marine invertebrate and plant systems. Advances in Marine Biology Vol 65, pp. 67-94.
Palumbi, S.R., 2003. Population genetics, demographic connectivity, and the design of marine reserves. Ecological applications 13, 146-158.
Pelc, R., Warner, R., Gaines, S., 2009. Geographical patterns of genetic structure in marine species with contrasting life histories. Journal of Biogeography 36, 1881-1890.
Pérez, M., Branco, M., Llavona, A., Ribeiro, P., Santos, A., Hawkins, S., Dávila, J., Presa, P., Alexandrino, P., 2007. Development of microsatellite loci for the black-footed limpet, *Patella depressa*, and cross-amplification in two other *Patella* species. Conservation Genetics 8, 739-742.
Pérez, M., Llavona, A., Presa, P., Ribeiro, P., Hawkins, S., Santos, A., Alexandrino, P., 2008. New polymorphic microsatellite markers for the limpet *Patella rustica* and cross- priming testing in four *Patella* species. Molecular ecology resources 8, 926-929.
Petit, R.J., El Mousadik, A., Pons, O., 1998. Identifying populations for conservation on the basis of genetic markers. Conservation biology 12, 844-855.
Philippart, C.J., Anadón, R., Danovaro, R., Dippner, J.W., Drinkwater, K.F., Hawkins, S.J., Oguz, T., O'Sullivan, G., Reid, P.C., 2011. Impacts of climate change on European marine ecosystems: observations, expectations and indicators. Journal of Experimental Marine Biology and Ecology 400, 52-69.
Pineda, J., Hare, J.A., Sponaungle, S., 2007. Larval transport and dispersal in the coastal ocean and consequences for population connectivity.
Pinn, E.H., Rodgers, M., 2005. The influence of visitors on intertidal biodiversity. Journal of the Marine Biological Association of the United Kingdom 85, 263-268.
Piraino, S., Fanelli, G., Boero, F., 2002. Variability of species' roles in marine communities: change of paradigms for conservation priorities. Marine Biology 140, 1067-1074.
Planes S, J.G., Thorrold SR 2009. Larval dispersal connects fish populations in a network of marine protected areas. Proceedings of the National Academy of Sciences 105, 5693-5697.
Planes, S., Jones, G.P., Thorrold, S.R., 2009. Larval dispersal connects fish populations in a network of marine protected areas. Proceedings of the National Academy of Sciences 106, 5693-5697.

Polunin, N., Roberts, C., 1993. Greater biomass and value of target coral-reef fishes in two small Caribbean marine reserves. Marine Ecology-Progress Series 100, 167-167.

Puillandre, N., Baylac, M., Boisselier, M.-C., Cruaud, C., Samadi, S., 2009. An integrative approach to species delimitation in *Benthomangelia* (Mollusca: Conoidea). Biological Journal of the Linnean Society 96, 696-708.

Putman, A.I., Carbone, I., 2014. Challenges in analysis and interpretation of microsatellite data for population genetic studies. Ecology and evolution 4, 4399-4428.

Ramajo, L., Baltanás, Á., Torres, R., Manríquez, P.H., Rodriguez-Navarro, A., Lagos, N.A., 2013. Geographical variation in shell morphology of juvenile snails (*Concholepas concholepas*) along the physical–chemical gradient of the Chilean coast. Journal of the Marine Biological Association of the United Kingdom 93, 2167-2176.

Ribeiro, P.M.d.A., 2008. Dispersal and connectivity of northeastern Atlantic patellid limpets: a multidisciplinary approach. University of Southampton.

Roberts, C.M., Andelman, S., Branch, G., Bustamante, R.H., Carlos Castilla, J., Dugan, J., Halpern, B.S., Lafferty, K.D., Leslie, H., Lubchenco, J., 2003. Ecological criteria for evaluating candidate sites for marine reserves. Ecological applications 13, 199-214.

Roberts, C.M., McClean, C.J., Veron, J.E., Hawkins, J.P., Allen, G.R., McAllister, D.E., Mittermeier, C.G., Schueler, F.W., Spalding, M., Wells, F., 2002. Marine biodiversity hotspots and conservation priorities for tropical reefs. Science 295, 1280-1284.

Rohlf, F.J., Archie, J.W., 1984. A comparison of Fourier methods for the description of wing shape in mosquitoes (Diptera: Culicidae). Systematic Biology 33, 302-317.

Rohlf, F.J., Marcus, L.F., 1993. A revolution morphometrics. Trends in Ecology & Evolution 8, 129-132.

Rosenberg, N.A., 2004. DISTRUCT: a program for the graphical display of population structure. Molecular Ecology Notes 4, 137-138.

Rossi, V., Ser- Giacomi, E., López, C., Hernández- García, E., 2014. Hydrodynamic provinces and oceanic connectivity from a transport network help designing marine reserves. Geophysical Research Letters 41, 2883-2891.

Rousset, F., 2008. genepop’007: a complete re- implementation of the genepop software for Windows and Linux. Molecular ecology resources 8, 103-106.

Russ, G.R., 2002. Yet another review of marine reserves as reef fishery management tools. Coral reef fishes: dynamics and diversity in a complex ecosystem. Academic Press, San Diego, California, USA, 421-443.

Sá-Pinto, A., Branco, M., Harris, D.J., Alexandrino, P., 2005. Phylogeny and phylogeography of the genus *Patella* based on mitochondrial DNA sequence data. Journal of Experimental Marine Biology and Ecology 325, 95-110.

Sá- Pinto, A., Baird, S.J., Pinho, C., Alexandrino, P., Branco, M., 2010. A three-way contact zone between forms of *Patella rustica* (Mollusca: Patellidae) in the central Mediterranean Sea. Biological Journal of the Linnean Society 100, 154-169.

Sala, E., Aburto-Oropeza, O., Paredes, G., Parra, I., Barrera, J.C., Dayton, P.K., 2002. A general model for designing networks of marine reserves. Science 298, 1991-1993.

Salm, R.V., Clark, J.R., Siirila, E., 2000. Marine and coastal protected areas: a guide for planners and managers. IUCN.
Schunter, C., Carreras-Carbonell, J., Planes, S., Sala, E., Ballesteros, E., Zabala, M., Harmelin, J.G., Harmelin-Vivien, M., Macpherson, E., Pascual, M., 2011. Genetic connectivity patterns in an endangered species: The dusky grouper (Epinephelus marginatus). Journal of Experimental Marine Biology and Ecology 401, 126-133.
Schwartz, M.K., Luikart, G., Waples, R.S., 2007. Genetic monitoring as a promising tool for conservation and management. Trends in ecology & evolution 22, 25-33.
Selkoe, K.A., Toonen, R.J., 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecology letters 9, 615-629.
Selkoe, K.A., Watson, J.R., White, C., Horin, T.B., Iacchei, M., Mitarai, S., Siegel, D.A., Gaines, S.D., Toonen, R.J., 2010. Taking the chaos out of genetic patchiness: seascape genetics reveals ecological and oceanographic drivers of genetic patterns in three temperate reef species. Mol Ecol 19, 3708-3726.
Silva, I.C., Mesquita, N., Paula, J., 2010. Lack of population structure in the fiddler crab Uca annulipes along an East African latitudinal gradient: genetic and morphometric evidence. Marine Biology 157, 1113-1126.
Starr, R.M., O'Connell, V., Ralston, S., 2004. Movements of lingcod (*Ophiodon elongatus*) in southeast Alaska: potential for increased conservation and yield from marine reserves. Canadian Journal of Fisheries and Aquatic Sciences 61, 1083-1094.
Steneck, R.S., Graham, M.H., Bourque, B.J., Corbett, D., Erlandson, J.M., Estes, J.A., Tegner, M.J., 2002. Kelp forest ecosystems: biodiversity, stability, resilience and future. Environmental conservation 29, 436-459.
Sundelof, A., Jonsson, P.R., 2012. Larval dispersal and vertical migration behaviour - a simulation study for short dispersal times. Marine Ecology-an Evolutionary Perspective 33, 183-193.
Teske, P.R., Forget, F.R.G., Cowley, P.D., von der Heyden, S., Beheregaray, L.B., 2010. Connectivity between marine reserves and exploited areas in the philopatric reef fish *Chrysoblephus laticeps* (Teleostei: Sparidae). Marine Biology 157, 2029-2042.
Thompson, R., Crowe, T., Hawkins, S., 2002. Rocky intertidal communities: past environmental changes, present status and predictions for the next 25 years. Environmental Conservation 29, 168-191.
Tlig-Zouari, S., Rabaoui, L., Fguiri, H., Diawara, M., Ben Hassine, O.K., 2011. Morphometric characterization of the limpet Patella ferruginea along the Tunisian coasts. CBM-Cahiers de Biologie Marine 52, 269.
Toonen, R.J., Andrews, K.R., Baums, I.B., Bird, C.E., Concepcion, G.T., Daly-Engel, T.S., Eble, J.A., Faucci, A., Gaither, M.R., Iacchei, M., 2011. Defining boundaries for ecosystem-based management: a multispecies case study of marine connectivity across the Hawaiian Archipelago. Journal of Marine Biology 2011.
Underwood, A., 2000. Experimental ecology of rocky intertidal habitats: what are we learning? Journal of Experimental Marine Biology and Ecology 250, 51-76.
Underwood, A.J., 1996. Experiments in ecology: their logical design and interpretation using analysis of variance. Cambridge University Press.

Van Bocxlaer, B., Schultheiß, R., 2010. Comparison of morphometric techniques for shapes with few homologous landmarks based on machine-learning approaches to biological discrimination. Paleobiology 36, 497-515.
Vaselli, S., Bertocci, I., Maggi, E., Benedetti-Cecchi, L., 2008. Assessing the consequences of sea level rise: effects of changes in the slope of the substratum on sessile assemblages of rocky seashores. Marine Ecology Progress Series 368, 9-22.
Venables, W.N., Smith, D.M., 2005. the R development core team. An Introduction to R. Notes on R: A Programming Environment for Data Analysis and Graphics.
Villamor A, C.F., Abbiati M (2014), 2014. Genetic Structuring across Marine Biogeographic Boundaries in Rocky Shore Invertebrates. PloS One.
Warner, R.R., Cowen, R.K., 2002. Local retention of production in marine populations: evidence, mechanisms, and consequences. Bulletin of Marine Science 70, 245-249.
Watters, J.V., Lema, S.C., Nevitt, G.A., 2003. Phenotype management: a new approach to habitat restoration. Biological Conservation 112, 435-445.
Weersing, K., Toonen, R.J., 2009. Population genetics, larval dispersal, and connectivity in marine systems. Marine Ecology Progress Series 393, 1-12.
Wernberg, T., Russell, B.D., Moore, P.J., Ling, S.D., Smale, D.A., Campbell, A., Coleman, M.A., Steinberg, P.D., Kendrick, G.A., Connell, S.D., 2011. Impacts of climate change in a global hotspot for temperate marine biodiversity and ocean warming. Journal of Experimental Marine Biology and Ecology 400, 7-16.
Will, K.W., Mishler, B.D., Wheeler, Q.D., 2005. The perils of DNA barcoding and the need for integrative taxonomy. Systematic biology 54, 844-851.
Williams, G.C., 2008. Adaptation and natural selection: a critique of some current evolutionary thought. Princeton University Press.
Williams, S.T., Hall, A., Kuklinski, P., 2012. Unraveling Cryptic Diversity in the Indo-West Pacific Gastropod Genus Lunella (Turbinidae) using Elliptic Fourier Analysis*. American Malacological Bulletin 30, 189-206.
Wood, A.R., Gardner, J.P.A., 2007. Small spatial scale population genetic structure in two limpet species endemic to the Kermadec Islands, New Zealand. Marine Ecology Progress Series 349, 159-170.
Zachos, F.E., Apollonio, M., Bärmann, E.V., Festa-Bianchet, M., Göhlich, U., Habel, J.C., Haring, E., Kruckenhauser, L., Lovari, S., McDevitt, A.D., 2013. Species inflation and taxonomic artefacts—A critical comment on recent trends in mammalian classification. Mammalian Biology-Zeitschrift für Säugetierkunde 78, 1-6.